University of Alberta

Maternal and Dietary Vitamin D Source Affect Chicken Hatchability, Production Performance, Bone Quality and Innate Immune Function

by

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ABSTRACT

Vitamin D is involved in calcium metabolism, reproduction, bone quality and immune function in chickens. The innate immune system of broilers is inefficient at hatch, making the chick susceptible to infection. Dietary 25-OH D_3 may be more available for conversion to $1,25(OH)_2D_3$ than vitamin D_3, thus enhancing vitamin D functions. Quantitative computed tomography (QCT) was evaluated as a method of assessing bone quality in poultry. Scan location and bone handling treatment within an experiment were important considerations for QCT use in poultry studies. The strongest relationship with QCT total bone mineral density was breaking strength, demonstrating that QCT is suitable for measurement of bone functional characteristics in poultry. Additionally, QCT allows a more in-depth analysis of bones than traditional methods, providing both trabecular and cortical bone measures. The effects of both direct and maternal supplementation of 25-OH D_3 on broiler production, bone quality and innate immune function were assessed. Broiler dietary 25-OH D_3 increased final body and *pectoralis* major weights, and enhanced bone quality relative to those fed vitamin D_3. During an inflammatory challenge, dietary 25-OH D_3 at 69 µg/kg + 2,500 U vitamin D_3, or at 103.5 µg/kg reduced effects on bone quality. Maternal 25-OH D_3 improved egg quality, hatchability and chick feed conversion. Maternal 25-OH D_3 increased the bactericidal activity of chick innate immune cells 4 d for early and mid-production hatches, respectively, and at 1 and 4 d for the late production hatch. This could partially be associated with the increased phagocytosis of *E. coli* by immune cells from the 25-OH D_3 chicks at 1 d in the late production hatch, although there were no differences in phagocytosis for the early and mid-production hatches. Oxidative burst of heterophils

from the 25-OH D_3 chicks was greater than the D_3 chicks at 1 d for the mid-production hatch. Overall, 25-OH D_3 improved production efficiency and bone quality of broilers, and maternal supplementation improved hatchability and early innate immune function of chicks. Supplementation with 25-OH D_3 could be a nutritional means to enhance bone quality, immunity and broiler production efficiency.

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LIST OF ABBREVIATIONS

$1,25(OH)_2 D_3$	1,25-dihydroxycholecalciferol
25-OH D_3	25-hydroxycholecalciferol
aP	Available phosphorus
APC	Antigen presenting cell
BW	Body weight
BMD	Bone mineral density
BSA	Bovine serum albumin
Ca	Calcium
CCAC	Canadian Council on Animal Care
CO_2	Carbon dioxide
ChIFN	Chicken Interferon
D_3	Cholecalciferol
CV	Coefficient of variation
CSF	Colony stimulating factor
CP	Crude protein
CTL	Cytotoxic T-lymphocyte
d	day (s)
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein diacetate
DDH_2O	Double distilled water
DEXA	Dual energy x-ray absorptiometry
D_2	Ergocalciferol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPD	External pip dead
EPL	External pip live
FCE	Feed conversion efficiency

h	hour (s)
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
IFN	Interferon
IL	Interleukin
IPD	Internal pip dead
IPL	Internal pip live
ICU	International chick unit
IU	International unit
IEL	Intraepithelial lymphocyte
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M-CSF	Macrophage colony stimulating factor
MIP	Macrophage inflammatory protein
MHC-II	Major histocompatibility class II
MPO	Myeloperoxidase
NRC	National Research Council
NK	Natural killer
NDV	Newcastle disease virus
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
PTH	Parathyroid hormone
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
P. Major	*Pectoralis major*
P. Minor	*Pectoralis minor*
PMA	Phorbol myristate acetate
PBS	Phosphate buffered saline

P	Phosphorus
PMN	Polymorphonucleated
QCT	Quantitative computed tomography
ROS	Reactive oxygen species
RBC	Red blood cell
SE	*Salmonella enteritidis*
SE-ILK	*Salmonella enteritidis*-immune T-cell lymphokine
SRBC	Sheep red blood cell
O_2^-	Superoxide
TD	Tibial dyschondroplasia
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UV	Ultraviolet
DBP	Vitamin D binding protein
VDR	Vitamin D receptor
wk	week (s)
WBC	White blood cell

Chapter 1: Literature Review

1. Introduction

1.1. Today's Broiler Chicken: Health and Skeletal Issues

There have been vast improvements in growth potential of the broiler chicken over the past several years; however this may have led to compromises in bone and immune function development (Lilburn, 1994; Bayyari, et al., 1997; Julian, 1998; Williams, et al., 2000; Yunis, et al., 2000). Currently, less than a third of the number of days is needed for the bird to reach market weight than 40 years ago (Havenstein, et al., 2003b). Along with faster growth rates, there has also be increased selection for a greater proportion of breast muscle (Lilburn, 1994; Havenstein, et al., 2003a). These changes, although of great benefit for the poultry production and processing industries, have the potential to lead to an increasing number of skeletal and health issues.

Nutrition is an important component of proper management for optimal growth and development of the bird. Nutrition plays a vital role in not only providing nutrients for growth, but also for all metabolic processes such as homeostasis, bone development, reproduction, and immune function. Poor nutrition leads to poor skeletal development and lameness and also makes birds more susceptible to disease and infection (Klasing, 1998b; Edwards, 2000; Kidd, 2004; Julian, 2005).

Vitamin D is a vital nutrient required for several bodily processes. Vitamin D is first metabolized in the liver to 25-hydroxycholecalciferol (25-OH D_3) and then in the kidney to its active form, 1,25-dihydroxycholecalciferol ($1,25(OH)_2$ D_3) (Soares, et al., 1995). Vitamin D is primarily known for its role in Ca metabolism and therefore its requirement

in bone formation and maintenance. However, more recently, vitamin D has been shown to be involved in the certain aspects of immune function. Its versatility in function has made vitamin D an interesting and challenging area of recent research in several animal species. Currently, 25-OH D_3 is available to use in poultry diets and has the potential to enhance the physiological roles of vitamin D.

1.2. Bone

1.2.1. Formation and Composition

There are two main types of bone found in all birds; cortical bone is the outer shell that provides most of the structural strength and trabecular bone, which is found within the cortical shell, formed in spicules, and also adds to the structural integrity of the bone (Whitehead and Fleming, 2000). In female birds, a third type of bone, medullary bone, forms when the hen nears sexual maturity in preparation for egg production (Whitehead and Fleming, 2000). Medullary bone acts as a labile Ca source from which the hen can draw upon when dietary Ca is insufficient during eggshell formation as evidenced by the high Ca turnover rate within this bone type (Hurwitz, 1965; Candlish, 1971). In several mammalian (Hodgskinson, et al., 1989) and fowl species (Bonser, 1995), cortical bone has been found to be stronger than trabecular bone and for the mammalian species this was attributed to the higher Ca content of the cortical bone (Hodgskinson, et al., 1989). In laying hens, the collagen content has been found to be greater in the cortical bone than the medullary bone (Knott and Bailey, 1999). However, medullary bone within the humerus has been found to contribute to bone strength in laying hens (Fleming, et al., 1998).

Skeletal formation begins early in embryonic life and continues throughout the life of the bird, continually being remodeled. At approximately 10 to 12 d of embryonic growth, calcium is mobilized from the shell, across the chorioallantoic membrane, through the circulatory system, to begin calcification of the skeleton of the developing chick (Tuan and Scott, 1977; Kubota, et al., 1981). There are three main bone cell types, these are known as the osteoblasts, osteocytes and osteoclasts, and are responsible for bone matrix formation, mineralization and bone resorption, respectively (Gay, et al., 2000; Rath, et al., 2000). Chondrocytes, bone building cells, proliferate at the growth plate on each end of the long bones (Farquharson and Jefferies, 2000). Bone mineralization starts of the mid section of the bone, where the chondrocytes become hypertrophic, mineral deposition occurs, replacing the cartilage matrix with bone, at which point the chondrocytes go through apoptosis (Velleman, 2000; Whitehead, 2004). Once the mid-bone region is calcified, this process continues in both directions towards the bone ends (Velleman, 2000).

Calcium and phosphorus form the majority of mineral matrix of the bone, which together form hydroxyapatite and make up about 70% of the bone weight (Rath, et al., 2000). Bone collagen is another major component of bone. It forms the majority (80-90%) of the organic matrix (Rath, et al., 2000). Collagen forms a lattice network of crosslinking fibrils that provide tensile strength to the matrix (Velleman, 2000).

There are differences in the bone mineral composition and amount of mineral depending on the area of the bone being examined. For example, the tibiotarsus has greater mineral content at the mid and distal end than the proximal end of the bone (Williams, et al., 2000). The femur midpoint of a 7 wk old broiler has a greater

microhardness test measure and % ash content than the distal end of the bone (Bonser and Casinos, 2003). It can therefore be concluded that bone mineral content is at its greatest in the midsection, which can have implications on the interpretation for bone quality measurements and how they relate to the overall bone integrity of the bone *in vivo* (Bonser and Casinos, 2003).

1.2.2. Bone quality problems

The rate of bone development does not match the fast growth rates of commercial broilers (Rath, et al., 2000; Leslie, et al., 2006). Bone ash content is lower and bone porosity is higher in growth-selected birds as compared to a slower growing control strain (Williams, et al., 2000). Bone formation is most rapid at 28 d of age (Leslie, et al., 2006) and maximum bone density and breaking strength is not reached until 35 wk of age in broilers (Rath, et al., 2000), long after birds are sent to processing which is often less than 42 d age. Several bone development and structural problems can arise from poorly formed bones (Lilburn, 1994; Thorp, 1994; Julian, 1998), causing bone breakage, impairing the welfare of the broiler, leading to culls, as well as carcass downgrades at processing.

Growth rate affects the way in which the bone is formed. With rapid growth rate in broilers, the ability to form a tight, compact bone matrix may be impaired, leading to pores within the bone matrix, which has been shown to weaken the bone structure (Thorp and Waddington, 1997; Williams, et al., 2000). Other skeletal problems related to the fast growth rate of commercial broilers are an increased incidence of tibial dyschondroplasia and angular bone deformities that can result in bone deformation over

time (Lilburn, 1994; Julian, 1998). Therefore, bone quality of broilers is of both welfare and economic concern affecting many aspects of the poultry industry, from the bird through to the processors.

1.2.3. Methods used for bone quality analysis

There has been considerable effort to better understand poultry bone metabolism. Breaking strength (Crenshaw, et al., 1981; Cheng and Coon, 1990; Elaroussi, et al., 1994a), ash (Clunies, et al., 1992; Cransberg, et al., 2001; Hall, et al., 2003) and Ca content determination (Clunies, et al., 1992; Cransberg, et al., 2001) are among the more commonly used methods in determining bone quality in poultry research. These methods, although useful for the study of bone quality, are limited in the information they provide. First, each of these procedures are not suitable for drawing conclusions on the functional differences among different bone fractions. Secondly, these procedures can only be performed *ex vivo*, therefore measurement of changes in bone quality throughout the life cycle requires a large number of birds to be killed at various times for bone analysis. Thirdly, assessing bone integrity through breaking strength may not accurately reflect bone integrity in vivo, as this test measures resistance to breakage caused by a force applied perpendicular to the bone. Lastly, bone removal and preparation techniques have been shown to affect certain bone quality measurements, such as ash, density, and strength measurements (Orban, et al., 1993). Using novel technologies, it is now possible to further investigate avian bone metabolism and function than can not be achieved using these destructive tests.

Bone mineral density (BMD) measured by Quantitative Computed Tomography (QCT) is currently used in the medical profession to monitor osteoporosis in humans in both research and clinical situations (Genant, et al., 1987; Wachter, et al., 2001). Through a series of multiple x-rays that are taken at many different angles around the entire bone, QCT calculates the true volumetric BMD, provided as a 3-dimenisional measurement of BMD and distribution (Korver, et al., 2004). The main advantages of QCT are that it is a noninvasive measure of BMD and allows for the separate measurement of BMD and areas of the cortical and trabecular bone regions (Genant, et al., 1987). For poultry research, this would mean fewer birds are needed throughout the trial for BMD analysis as birds can simply be sedated for the analysis procedure without the need to sacrifice the bird for each sample time point. In addition, BMD and area at multiple sites along the bone can be determined.

Studies comparing various bone quality techniques in humans and animals have shown QCT to be comparable with other BMD and bone quality measurements. QCT has been shown to yield similar results as histomorphometry (Rosen, et al., 1995), dual energy radiography in healthy and osteoporotic subjects (Pacifici, et al., 1990), bone ash (Genant, et al., 1987; Waite, et al., 2000), bone Ca (Markel, et al., 1991), lateral and posteroanterior dual x-ray absorptiometry (Genant, et al., 1987; Guglielmi, et al., 1994), and spinal fracture index (Genant, et al., 1987). To date, QCT has not been adequately assessed for use in poultry bone research although its proven advantage in other species makes it an ideal technology to study poultry bone metabolism.

1.3. Innate Immune System

1.3.1. Overview of the Innate Immune System

The initiation of the innate immune defense causes the release of factors resulting in not only killing of the invading pathogen but also muscle degradation, decreased feed intake and therefore stunted growth of the bird (Mireles, et al., 2005). These are not favorable attributes in poultry production. An increased understanding of immune function in poultry may help in efforts to find ways to achieve the optimum immune response that is one that minimizes the destructive effects it has on growth of the bird.

Heterophils, monocyte/macrophages and natural killer cells make up the majority of cellular components of the innate (non-specific) immune response (Powell, 1987b). These cells are the first line of defense in an inflammatory immune response. Innate immune cells are responsible for recognizing pathogens and launching an attack to effectively kill the invading pathogen. In addition, macrophages are antigen presenting cells and have an impact on how fast the T-cells respond to an infectious challenge (Morrissette, et al., 1999).

Birds can be exposed to pathogenic organisms as early as the initial stages of embryo development, therefore early immune system development and function is important in protecting the growing embryo and newly hatched chick. Current research on the innate immune response has determined many of the cellular mechanisms involved, with hopes of understanding avian immune function in current commercial strains of broilers.

1.3.2. Innate Immune Cells and Function

1.3.2.1. Heterophils

1.3.2.1.1. Heterophil Morphology and Mammalian Neutrophil Comparision

The heterophil is a polymorphonucleated (PMN; usually 2-3 lobes) white blood cell (Stabler, et al., 1994), which originates from the bone marrow. The heterophil is described as the avian equivalent to the mammalian neutrophil. Although they function in a similar manner, there are some key differences between the avian heterophil and the mammalian neutrophil reported in the literature. Heterophils differ in granule content from those present in the mammalian neutrophil. It was initially believed that heterophil granules did not contain myeloperoxidase (MPO) (Brune and Spitznagel, 1973; Penniall and Spitznagel, 1975). Myeloperoxidase is an important enzyme used in oxygen-dependent killing of microorganisms in neutrophils of many different species, including humans (Styrt, 1989). However, a more recent report by Lam (1997) found measurable MPO activity and isolated a gene similar to that of the human MPO gene from the avian heterophil. Avian heterophil granules also do not contain peroxidase, alkaline phosphatase or catalase (Brune and Spitznagel, 1973; Daimon and Caxton-Martins, 1977; Powell, 1987a). Secondly, the avian heterophil does not produce a strong oxidative burst, unlike its mammalian counterpart (Desmidt, et al., 1996). A strong oxidative burst (an oxygen-dependent killing mechanism) cannot be launched with the lack of or low levels of MPO.

1.3.2.1.2. Heterophil Function in the Inflammatory Immune Response

Heterophils play an immediate and important role in the inflammatory immune response. As the heterophil is one of the first cells to respond to an invading pathogen,

birds with a greater level of peripheral heterophils are better able to defend against invading pathogens (Kogut, et al., 1994a; Kogut, et al., 1994b). During an inflammatory response, a large number of heterophils migrate to the site of infection within the first 5-12 hours, during the acute phase of inflammation (Jortner and Adams, 1971; Kogut, et al., 1994a; Kogut, 2002; Petrone, et al., 2002; Van Immerseel, et al., 2002). Heterophils are not only one of the first cell types to migrate to the site of infection but have been found to constitute upwards of 80% of the migrated leukocytes (Kogut, et al., 1994a). This rapid response of heterophils to the site of infection indicates their early protective role in the inflammatory immune response.

Heterophil function varies among strains of chickens and is an inherited trait passed on to the offspring (Swaggerty, et al., 2003). Chicks with genetically more efficient heterophils are less susceptible to *Salmonella enteritidis* (SE) infection and tissue SE invasion than those with genetically less efficient heterophils (Ferro, et al., 2004; Swaggerty, et al., 2005). The ability to efficiently defend against pathogen invasion could mean fewer resources (energy and nutrients) are needed to fight the infection, potentially decreasing the harmful effects of an immune response on growth and production of commercial birds. The growth potential of these "heterophil efficient" birds as compared to the less heterophil efficient strains was not reported. However in most studies, growth rate and production performance have been inversely related to immunological competence (Yunis, et al., 2000; Cheng, et al., 2001; Yunis, et al., 2002).

1.3.2.1.3. Heterophil Migration, Activation and Bacterial Recognition

Local production and release of chemotatic mediators stimulate the migration of heterophils to infected tissue (Kogut, et al., 1995a). Chemoattractants have not been well studied in the chicken; however, the actual invasion process by bacteria has been shown to initiate the migration process (Kogut, et al., 1995a). Heterophils are activated by cytokines as well as chemokines. Cytokines are messenger proteins that are produced by immune cells to initiate and stimulate the immune response (Grimble, 1998). Interleukins (IL), interferons (IFN), colony stimulating factors (CSF), tumor necrosis factors (TNF) and transforming growth factors (TGF) are all types of cytokines (Grimble, 1998). Chemokines are small pro-inflammatory proteins that act as chemoattractants for leukocytes, in the chicken these are CXC, CC and C chemokines (Magor and Magor, 2001; Staeheli, et al., 2001). In addition, lymphokines are cytokines that are secreted by activated lymphocytes (both T and B-cells) (Schattner, 1994).

In human bacterial-infected epithelial cells, IL-8 is released, which is a PMN chemoattractant in humans (Eckmann, et al., 1993). Recently an IL-8-like factor has been shown to act as a chemotactic mediator of heterophils in the peritoneum of chicks injected with SE-immune T-cell lymphokines (SE-ILK) and SE (Kogut, 2002). The process of obtaining SE-immune T-cell lymphokines involves immunizing birds with SE, then collecting T-cells from harvested spleens of those hens. The T-cells are then incubated with concanavalin A, after 48 h cells are removed and Con A is inactivated and the supernatants containing the SE-ILK are concentrated to use as a prophylactic treatment in chicks (Tellez, et al., 1993). Furthermore, IL-8 expression has been shown to be up-regulated in a line of *Salmonella*-resistant chickens (Swaggerty, et al., 2004).

This may suggests a similar role of IL-8 as a chemoattractant factor of heterophils in the chicken.

Heterophils recognize pathogens through pattern recognition receptors (PRR) on their surface, which interact with pathogen-associated molecular patterns on bacteria (PAMP) (Farnell, et al., 2003b). The PAMP on the surface of bacteria are essential components of the bacteria, such as the carbohydrates and protein structures, and are therefore not likely to mutate (Aderem, 2002; Aderem and Smith, 2004). Therefore, these structures are able to be recognized by PRR such as Toll-like receptors (TLR), scavenger receptors, complement receptors, C-type lectin receptor and integrins (Aderem and Smith, 2004).

Toll-like receptors are used by phagocytic cells to recognize several PAMP on bacteria (Janeway and Medzhitov, 2002; Aderem and Smith, 2004). Researchers have discovered eleven distinct TLR in humans and mice, some of which have been reviewed by Janeway and Medzhitov (2002). Each TLR recognizes different components of the bacteria (Farnell, et al., 2003b; Aderem and Smith, 2004). Research into chicken TLR is very recent and to date 13 chicken TLR genes from different tissues and immune cells have been described (Fukui, et al., 2001; Iqbal, et al., 2005a; Iqbal, et al., 2005b; Kogut, et al., 2005; Yilmaz, et al., 2005; Temperley, et al., 2008).

Toll-like receptors have been shown to mediate immune responses in poultry. Using goat polyclonal antibodies raised against rat CD14 and human TLR2 and TLR4, Farnell et al. (2003a, 2003b) demonstrated that oxidative burst in response to lipopolysaccharide (LPS) and *Staphylococcus aureus* lipoteichoic acid (LTA) in chicken heterophils was mediated through TLR. Specific TLR were shown to mediate specific

heterophil oxidative burst responses towards different pathogens. Toll-like receptor-2 and TLR4 were involved in LPS-stimulated, and TLR2 and CD14 were involved in LTA-stimulated oxidative burst (Farnell, et al., 2003b). Similarly, the TLR4 gene in mice is necessary for an LPS immune defense (Poltorak, et al., 1998; Qureshi, et al., 1999; Takeuchi, et al., 1999). This indicates TLR4 may play a similar role in murine and avian species. In mammals, TLR4 also plays a role in the recognition of LTA (Takeuchi, et al., 1999), which was not found to be the case for the chicken (Farnell, et al., 2003b). In mammals, TLR2 recognizes several different ligands (Janeway and Medzhitov, 2002). In mice, TLR2 does not seem to be involved in LPS recognition but necessary in the recognition of several gram positive bacteria (Takeuchi, et al., 1999). The development of chicken polyclonal TLR antibodies would further clarify these relationships in birds.

1.3.2.1.4. Heterophil phagocytic process

Phagocytosis is the process by which antigens are engulfed by the phagocytic cell, to be killed by subsequent mechanisms. In mammals, phagocytosis is activated by many cell surface receptors on the leukocyte, these include the classical ones such as Fc and complement receptors, as well as integrins, CD14, scavenger receptors, and TLR (Allen and Aderem, 1996; Ross and Auger, 2002; Underhill and Ozinsky, 2002). Once the antigen is bound, the immune cell membrane is extended to surround the antigen and the particle is then ingested forming a phagosome (Allen and Aderem, 1996; Goldsby, et al., 2000). The phagosome matures, fuses with the lysosome and forms a phagolysosome (Allen and Aderem, 1996; Goldsby, et al., 2000). This elicits killing mechanisms within the phagocytic cells, which are discussed in more detail in the following sub-sections.

Signaling pathways for heterophil bacterial phagocytosis have been studied to a limited extent. The classical receptors, Fc and complement, as well as TLR have been studied in chicken heterophil phagocytosis (Kogut, et al., 2001a; Kogut, et al., 2001b; Kogut, et al., 2005). Studies to date indicate that G-proteins mediate heterophil phagocytosis, and that Ca^{++} also acts as a signal involved in the phagocytosis of serum-opsonized (complement receptor-mediated phagocytosis) and IgG-opsonized (Fc receptor mediated phagocytosis) *Salmonella* (Kogut, et al., 2001a; Kogut, et al., 2001b). In addition, Fc mediated phagocytosis is also mediated by protein kinase C (Kogut, et al., 2001b).

Heterophil phagocytosis capability improves with age in chicks (within the first week post-hatch) (Wells, et al., 1998). This improvement was found be the result of the maturation of heterophils capable of phagocytosing bacteria (Wells, et al., 1998). Heterophil phagocytosis was also shown to be age-dependent in turkey poults, with much greater phagocytosis and killing capability in the 2nd and 3rd week post-hatch than the 1st (Lowry, et al., 1997).

1.3.2.1.5. Heterophil mechanisms in bacterial killing

Once phagocytosed, heterophils can kill bacteria through two different mechanisms, oxidative burst, through the production of reactive oxygen species (ROS) and degranulation, through the actions of granules (which are located within the heterophil and contain enzymes and antimicrobial peptides) in response to the phagocytosis of an invading pathogen within the heterophil (Powell, 1987a). These methods are discussed in detail in the following sub-sections.

1.3.2.1.5.1. Heterophil oxidative burst in inflammatory response

Heterophils will react with an oxidative burst in response to a phagocytosed pathogen to attempt to kill the invading pathogen. This process has been described for neutrophils, where metabolized glucose and nicotinamide adenine dinucleotide phosphate (NADPH) formation increase oxygen consumption by the NADPH-oxidase enzyme complex, resulting in the formation of superoxide (O_2-), which then generates various reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (Borregaard, 1988; Dahlgren and Karlsson, 1999). Hydrogen peroxide and hydroxyl radicals have antimicrobial activity in the presence of MPO (Klebanoff, 1970), a granule which has been discovered to exist in the chicken, although at low levels (Lam, 1997).

The oxidative burst response of chicken heterophils has been shown to be much lower than that in other species. Heterophils produce significantly fewer oxygen radicals than bovine neutrophils (Desmidt, et al., 1996). Stabler et al. (1994) found no increase in $O_2^{\cdot}$ production during the phagocytosis of *Salmonella* by chicken heterophils, suggesting an alternate (non-oxygen related) killing mechanism may be more important in the killing of bacteria by heterophils. Past research has shown that chicken heterophils go through an oxidative burst and oxidize glucose, however they do not produce hydrogen peroxide (H_2O_2) (Penniall and Spitznagel, 1975).

1.3.2.1.5.2. Heterophil degranulation response in bacterial killing

Degranulation is the release of enzymes and antimicrobial peptides from phagocytic cell granules in response to the phagocytosis of an invading pathogen.

Degranulation causes changes in heterophil shape and a reduction in density of the cytoplasmic granules (Rath, et al., 1998). Heterophil granules have different contents than mammalian neutrophils (reviewed by (Harmon, 1998), and therefore may rely on different degranulation killing mechanisms than mammalian neutrophils. There are at least two different types of granules (maybe 3) within the avian heterophil; the 3rd may have been from contaminating cells (Brune and Spitznagel, 1973). A few years later, using electron microscopy, Daimon and Caxton-Martins (1977) were able to identify three types of granules present in the chicken heterophil. The most prominent of these was the largest of the three, termed heterophil Type I, which accounted for 48.3% of the granules (Daimon and Caxton-Martins, 1977). Heterophil Type I granules do not contain peroxidase enzyme or alkaline phosphatase, but do have cationic peptides, lysozyme and acid phosphatase (Brune and Spitznagel, 1973; Daimon and Caxton-Martins, 1977). Type I granules have a strong acid phosphatase activity and are considered true lysosomes (Daimon and Caxton-Martins, 1977). Heterophil Type 1 granule contents were able to inhibit the growth of bacteria (Brune and Spitznagel, 1973), illustrating an antimicrobial effect. The mechanism of action of the smaller heterophil granules (Types II and III) is unknown (Daimon and Caxton-Martins, 1977).

The degranulation ability of heterophils from the *Salmonella*-resistant and -susceptible chicken lines were different with the *Salmonella*-resistant line releasing a greater amount of β-D-glucuronidase *in vitro* (Swaggerty, et al., 2003). Beta-D-glucoronidase is an acid hydrolase that is released during degranulation by the heterophil granules to kill pathogens (Brune and Spitznagel, 1973; Harmon, 1998). This increases

their ability to effectively kill invading pathogens that have been successfully phagocytosized by the heterophil.

1.3.2.1.6. Bacterial killing potential of the avian heterophil

Similar to heterophil phagocytosis, heterophil killing capabilities have been shown to be age-dependent in the chick (from 1 to 7 d) and turkey poult (from 1 to 14 d) (Lowry, et al., 1997; Wells, et al., 1998). Research has shown that the development of the killing ability can be manipulated through early exposure to SE- immune lymphokines (Kogut, et al., 1995b; Lowry, et al., 1997; Genovese, et al., 1999).

1.3.2.2. Macrophages

1.3.2.2.1. Monocyte/Macrophage Development and Morphology

Macrophages go through three differentiations before becoming mature. In the bone marrow, chicken stem cell monoblasts, with the aid of macrophage colony stimulating factor (M-CSF), develop into pro-monocytes and then to monocytes (Qureshi, et al., 2000; Qureshi, 2003). Monocytes are large round cells with a bean-shaped nucleus and are the immature form of macrophages, which circulate within the peripheral blood (Ross and Auger, 2002). Monocytes will mature into macrophages and migrate into tissues, such as the spleen, liver and almost every other organ when they detect invading pathogens (Stabler, et al., 1994; Qureshi, 2003). Macrophages are larger than monocytes, have a bean-shaped nucleus, but are irregular in overall shape (Ross and Auger, 2002). Macrophages remain active in tissues for about 5 wk (Powell, 1987b). Chicken macrophages can further differentiate to form multinucleated giant cells (Jortner and

Adams, 1971), which are involved in the tissue repair process following an inflammatory response (Klasing, 1998a). Giant cells form an epithelial cell layer surrounding necrotic tissue (Jortner and Adams, 1971). Monocyte-like cells can also differentiate into osteoblasts, Kupffer cells of the liver, and Langerhans cells of the epidermis (Powell, 1987b; Klasing, 1998a; Qureshi, 2003).

1.3.2.2.2. Macrophage function in the inflammatory immune response

Chicken macrophages participate in regulation of the inflammatory immune response through cytokine production, as well as ingesting and killing bacteria and tumor cells (Qureshi, 2003). Chicken macrophages also function as antigen presenting cells (APC), thus participating in initiation of cellular immunity (Morrissette, et al., 1999; Qureshi, 2003). Macrophages, after degrading antigens, present bacterial peptides through MHC class I or II to B and T-cells (Qureshi, 2003). This is an example of how the innate and cellular immune systems function together.

Macrophages are one of the primary innate immune cell types involved early in the inflammatory response. After 6 hr post-inflammatory stimulation with turpentine (Jortner and Adams, 1971), and 3 hr after *S. enteritidis* injection (Van Immerseel, et al., 2002), monocyte and macrophage populations start to increase in the infected areas. This early invasion allows for the activation of these immune cells and release of cytokines that further signal additional immune responses.

1.3.2.2.3. Inflammatory monocyte/macrophage tissue recruitment and bacterial recognition

Through chemotaxis, monocytes are drawn to the site of inflammation, and mature into macrophages within infected tissues (Qureshi, et al., 2000). For phagocytosis to proceed, the binding of bacteria to specific macrophage surface receptors occurs first. Much research on human and rodent macrophage receptor-mediated bacteria binding has been done to date, whereas little has been reported for the chicken. Several mammalian surface receptors have been identified; these include Fc receptors, complement receptors (Aderem and Underhill, 1999), mannose receptor (Fraser, et al., 1998), scavenger receptors (Pearson, 1996; Peiser, et al., 2000), and TLR (Akira, et al., 2001). Avian macrophages bind bacteria ligands through both non-specific and receptor-mediated binding (Qureshi, et al., 2000). Chicken macrophages have a specific receptor, the mannose binding protein, for mannose and fructose that will bind antigens expressing these structures (Epstein, et al., 1996; Qureshi, 2003). Scavenger receptors are also present on macrophages (Qureshi, 2003). Chicken macrophages, similar to the heterophil, also utilize Fc receptors which recognized opsonized targets and complement receptors for complement coated targets (Qureshi, 2003).

In the mammalian macrophage, once the antigen is attached to the macrophage PRR (TLR2), the macrophage secretes TNF-α, which then mediates the immune response (Underhill, et al., 1999). TNF-α has not been found in the chicken, however, a TNF-like factor has been reported in LPS-stimulated MQ-NCSU macrophages (Rautenschlein, et al., 1999). This TNF-like factor stimulated NO production.

1.3.2.2.4. Monocyte/macrophage participate in antigen phagocytosis

Phagocytosis by macrophages proceeds in the same manner as that outlined for heterophils in Section 1.3.2.1.4. Macrophage phagocytic capability begins in the chicken embryo (Powell, 1987b). During an inflammatory insult, macrophages are initially immature and less capable of phagocytosis than macrophages elicited several hours post-infection (Chu and Dietert, 1988). Monocytes are less efficient at phagocytosis of bacteria than heterophils and unlike heterophils, the opsonization of the *Salmonella enteritidis* does not enhance phagocytosis by monocytes (Stabler, et al., 1994). Opsonization is the process by which a bacteria is coated in antibodies recognized by the immune cells of the chicken. This is likely due to the immaturity of monocytes until differentiated into mature macrophages.

Similar to heterophils, an age-dependent increase in phagocytic ability has been reported in macrophages (Qureshi, et al., 2000). There were a greater percentage of phagocytic macrophages and total number of sheep red blood cells (SRBC) phagocytosed by phagocytic macrophages at 7 vs 3 d of age (Qureshi, et al., 2000).

1.3.2.2.5. Pathogen Killing by Macrophages: Capability

The ability of monocytes to kill bacteria has been shown to be greatly increased through opsonization of the bacteria (Stabler, et al., 1994). Monocytes were only capable of killing 15% of non-opsonized as compared to 95% of opsonized bacteria within 120 minutes (Stabler, et al., 1994). Macrophages are very efficient at killing phagocytosed bacteria. Macrophages killed more than 80% of the phagocytosed bacteria within a short amount of time (Qureshi, et al., 1986; Kramer, et al., 2003). The ability of macrophages to kill ingested bacteria has also been shown to differ among different broiler chicken

lines (Qureshi and Miller, 1991). The elicitation of peritoneal macrophages in response to Sephadex injection and their ability to phagocytose opsonized SRBC is different among strains of broilers (Qureshi and Miller, 1991).

1.3.2.2.6. Macrophage oxygen-dependent pathogen killing

Similar to heterophils, macrophages also kill via a respiratory burst, through the production of ROS (Dietert and Golemboski, 1998). Enzymes present in the lysosome act on the pathogen when the phagosome and lysosome fuse to form a phagolysosome (Powell, 1987b). Pathogen killing is accomplished in the macrophage through production of ROS and hydroxyl radicals (Powell, 1987b; Heale and Speert, 2002). Reactive oxygen species destroy bacterial membranes and DNA (Hampton, et al., 1998).

1.3.2.2.7. Macrophage nitric oxide production in pathogen killing

In the macrophage, inducible nitric oxide synthase (iNOS) oxidizes L-arginine to L-citrulline, which results in the production of reactive nitrogen intermediates (MacMicking, et al., 1997; Dietert and Golemboski, 1998; Lillehoj and Li, 2004). Due to the lack of the urea cycle in the bird, arginine is not synthesized by the chicken as is the case in mammals and a dietary source is required (Cheeke, 1991).

Only stimulated macrophages produce nitric oxide (NO) (Hussain and Qureshi, 1997). Production of NO by macrophages varies *in vitro* depending on the macrophage source and stimuli used. Lillehoj and Li (2004), examining the MQ-NCSU and HD11 macrophage cell lines, found that the former cell line produced more NO in response to *E.coli* or LPS as compared to the HD11 macrophages, which produced higher amounts of

NO in response to IFN-γ. Crippen et al., (2003) found stronger nitrate production from the HD11 macrophage cell line than from monocytes or heterophils in response to killed Salmonella and recombinant chicken INF-γ. Using an iNOS inhibitor, this nitrate production was decreased, thereby indicating that iNOS was a major factor in the production of nitrate (Crippen, et al., 2003). Monocytes are also capable of producing the NO metabolite, NO_2^-, in response to bacteria (gram-negative and –positive), as well as LPS, and recombinant chicken IFN-γ (Crippen, et al., 2003).

There are strain differences in chickens with regards to NO production in response to antigens such as parasite and bacterial infections (Hussain and Qureshi, 1997; Dil and Qureshi, 2002; Lillehoj and Li, 2004). The increased NO production is correlated with an increased iNOS mRNA expression in chickens (Hussain and Qureshi, 1997; Dil and Qureshi, 2002).

1.3.2.3. Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes that originate from the bone marrow (Sharma, 1997; Goldsby, et al., 2000). Natural killer cells are cytotoxic cells which function to lyse virus and tumor cells without specific antigen stimulation, thereby providing a non-specific natural immunity, making them part of the innate immune system (Sharma, 1997; Erf, 2004; Fairbrother, et al., 2004). Although, NK cells are similar to cytotoxic T-cells (CTL) in their cytotoxic actions towards viruses, they differ functionally from CTL. NK cell do not express surface CD3 (Gobel, et al., 1994), there is no MHC requirement in virus recognition, and the NK immune response does not result in memory (Herberman and Ortaldo, 1981; Goldsby, et al., 2000). Natural killer

cells have been characterized within mammals; however, due to the lack of an antibody marker for the avian NK cell they have not been well chatacterized within the bird (Gobel, 2000).

In general, NK cell cytotoxicity increases with age of the bird, not reaching its maximum potential until after 6-7 wk of age (Sharma and Coulson, 1979; Lillehoj and Chai, 1988). Genetic differences and bird age influence NK cell activity of the chicken (Sharma and Coulson, 1979; Lillehoj and Chai, 1988).

1.3.2.3.1. NK cell killing mechanisms

Natural killer cells are similar to cytotoxic T-cells in their method of killing. However, unlike cytotoxic T-cells, NK cells do not need to be activated by antigens to express granules and therefore are always cytotoxic (Powell, 1987a; Goldsby, et al., 2000). To date the granular content and killing mechanisms have been reported to a limited extent for the chicken. Through visual assessment of the avian NK cell, using transmission electron microscopy, no visible granules were seen (Sieminski-Brodzina and Mashaly, 1991). However, Gobel et al. (1994), were able to identify granules within the avian NK cell, although these authors did note that the presence of these granules were limited.

1.3.3. Cytokines involved in the inflammatory immune response

When a pathogen is recognized and phagocytosed by PMN, released cytokines initiate and control the type of immune response through signaling among immune cells, as well as initiate acquired immunity (Grimble, 1998; Lowenthal, et al., 2000; Kaiser, et

al., 2004). There are several groups of cytokines, such as the INF, IL, CSF, TNF, chemokines, and lymphokines (Grimble, 1998; Staeheli, et al., 2001; Kaiser, et al., 2004). Cytokines can also be divided into pro- and anti-inflammatory cytokines. Interleukins are cytokines produced by and act on leukocytes (Kaiser, et al., 2004). In mammals, many IL (or IL-like activity) have been identified, several of which have also been identified in the chicken as well, such as IL-1, IL-2, IL-15, IL-6, IL-8, IL-16, IL-17, IL-18 (Staeheli, et al., 2001; Kaiser, et al., 2004).

Cytokines are vital in the maturation and function of PMN cells (Galligan and Yoshimura, 2003). Heterophils produce the pro-inflammatory cytokines IL-6, IL-8 and IL-18 (Swaggerty, et al., 2004). The importance of cytokines for chicken heterophil maturation and function has been demonstrated through the administration of SE-ILK *in ovo* or to day-old chicks in several studies (Kogut, et al., 1994a; Kogut, et al., 1995a; Kogut, et al., 1995b; McGruder, et al., 1995a; McGruder, et al., 1995b; Kogut, et al., 1997; Lowry, et al., 1997; Genovese, et al., 1998; Kogut, et al., 1998). These studies have shown reductions of *in vivo Salmonella* organ invasion, increased heterophil numbers at the site of infection, as well as overall increases in bacterial phagocytosis and killing through the administration of SE-ILK. Characterization of the SE-ILK indicated that a granulocyte-CSF-like (GM-CSF-like factor) was the main component responsible for the increased innate immune response (Kogut, et al., 1997).

An increased mRNA expression of the pro-inflammatory cytokines, IL-6, IL-18 and IL-8, and a decrease in anti-inflammatory cytokine, TGF-β4, has been reported in SE-resistant chickens (Ferro, et al., 2004; Swaggerty, et al., 2004). This pattern of mRNA cytokine expression was observed for SE, serum-opsonized-SE, and IgY-

opsonized-SE stimulated heterophils (Swaggerty, et al., 2004). Heterophils produce IL-6 in response to LPS stimulation (Rath, et al., 1998). The pro-inflammatory role of IL-6 in the inflammatory immune response of heterophils is therefore evident from these studies indicating heterophil production and upregulated mRNA expression to combat bacterial invasion. In addition, IL-8 acts as a chemotactic factor for heterophils during an SE infection (Kogut, 2002).

When activated by phagocytosed pathogens, macrophages release various pro-inflammatory cytokines (Trinchieri, 1997). Activated macrophages release IL-1, TNF-like factor and CSF (Rautenschlein, et al., 1999; Glick, 2000; Qureshi, 2003). Greater chicken IL-1β, the chemokine macrophage inflammatory factor-1β, IFN-γ, and iNOS mRNA expression resulted in birds after coccidiosis infections (Laurent, et al., 2001). Chicken TNF-like factor was found to have similar functions as the mammalian TNF although the structure is different (Rautenschlein, et al., 1999). Chicken TNF-like factor exhibited cytotoxic activity, causing macrophage NO production and physical changes in the MQ-NCSU macrophage cell line (Rautenschlein, et al., 1999).

Another cytokine involved with the production of NO by avian macrophages is IFN. Nitric oxide production in turkey macrophages is dependent on both stimulation and IFN (Suresh, et al., 1995). These authors also demonstrated a potential pro-inflammatory role of turkey IFN (Suresh, et al., 1995). Chicken interferon-γ (ChIFN-γ) induces the production of NO in both HD11 and MQ-NCSU chicken macrophage cell lines (Yeh, et al., 1999). ChIFN-γ also appears to elicit an antiviral effect (Song, et al., 1997; Yeh, et al., 1999). In addition, the treatment of birds with ChIFN-γ to coccidiosis

infected broiler chickens resulted better growth performance post-infection (Lowenthal, et al., 1997).

The avian macrophage produces chemokines that have a chemotactic function in the inflammatory immune response (Klasing, 1998a). These chemokines have been identified as macrophage inflammatory proteins 1 and 2 (MIP-1 and MIP-2) (Klasing, 1998a). When monocytes and macrophages were infected with *Mycoplasma gallisepticum*, they attracted more heterophils than uninfected cells (Lam and DaMassa, 2000; Lam, 2002). The chemokine causing the increased heterophil infiltration was determined to be a macrophage inflammatory protein, MIP-1β-like chemokine (Lam and DaMassa, 2000; Lam, 2002).

1.3.4. The importance of immune function in poultry

The ability of the chicken to combat a disease or infection successfully and efficiently is critical to minimize production losses. Certainly, through management practices the spread of disease and exposure to bacteria and viruses can be minimized, however it is unlikely that disease will ever be entirely eliminated from the poultry industry. An immune response to a bacterial insult can cause several destructive effects as the bird responds to the infection. Infections often lead to poorer feed conversion, decreased BW and bone strength (Mireles, et al., 2005) as well as muscle degradation in chicks because of the altered systemic metabolism caused by the inflammatory response used by the chick to kill the invading pathogen (Klasing and Johnstone, 1991; Klasing, 1998a; Mireles, et al., 2005). Therefore, even if the chick is able to successfully combat the infection in terms of survival, several economically important factors can be severely

hindered. In addition, commercial broilers and turkeys are genetically selected for fast growth rate. Some reports indicate that this has had a negative impact on the immune response, making modern birds more susceptible to infections than in the past (Bayyari, et al., 1997; Yunis, et al., 2000). The group of chickens most at risk for infection and disease is the young, newly hatched chick (<1 wk of age) as various aspects of the immune system are not mature at this young age (Lowenthal, et al., 1994; Wells, et al., 1998). Although the research presented in the following chapters does not look at all of the mechanisms involved in the immune response as presented in this literature review, they are still important to consider as they are involved in the innate immune response as a whole and could contribute to the interpretation of the current research.

1.4. Vitamin D

1.4.1. Vitamin D Metabolites

There are two important forms of vitamin D that occur naturally, ergocalciferol (D_2) and cholecalciferol (D_3) (Soares, et al., 1995). However, in the chick, vitamin D_3 has ten times the biological activity than D_2 (Chen and Bosmann, 1964), and therefore vitamin D_3 is the most important form of this vitamin important in the avian species.

Vitamin D_3 is metabolically inactive and needs to be converted to its hormonal form to carry out its functions within the body. Vitamin D_3 is first obtained by the bird either through UV light reaction with skin cholesterol or through the diet (Soares, et al., 1995). The metabolism of vitamin D to its active form occurs through two main conversions. First, vitamin D_3 is hydroxylated in the liver by the actions of 25-hydroxylase on carbon 25 to yield 25-hydroxychocalciferol (25-OH D_3) (Soares, et al., 1995; Brown, et al.,

1999). Levels of plasma 25-OH D_3 are sensitive to dietary levels of vitamin D_3 (Brown, et al., 1999) and plasma levels are considered a good indication of vitamin D_3 status of the bird as it is the major circulating vitamin D_3 metabolite (Haussler and Rasmussen, 1972).

Twenty-five-OH D_3 is then hydroxylated to $1,25(OH)_2 D_3$ in the kidney by 25-hydroxy-D_3-1α-hydroxylase (Norman and Hurwitz, 1993). $1,25(OH)_2D_3$ is a steroid hormone (Norman, 1968), and it is through this final metabolite that vitamin D exerts its actions on Ca metabolism and cellular differentiation (Norman and Hurwitz, 1993). The synthesis of $1,25(OH)_2D_3$ from 25-OH D_3 also occurs in mammalian macrophages, although under different regulation than the renal production of $1,25(OH)_2D_3$ (Cohen and Gray, 1984; Overbergh, et al., 2000).

1.4.2. Vitamin D Metabolism

In the chicken, the intestinal absorption of vitamin D_3 and 25-OH D_3 takes place in the upper part of the jejunum (Bar, et al., 1980). Dietary vitamin D_3 is absorbed from the intestine with lipid-bile salt micelles into the lymph, then into the bloodstream where it is transported to other areas such as the liver, spleen, and kidneys (Leeson and Summers, 2001). However, it has been proposed that 25-OH D_3 is less reliant on the fat micelle to be absorbed into the intestine (Compston, et al., 1981). In humans with fat absorption deficiencies, the absorption of 25-OH D_3 was unchanged from normal patients, while vitamin D absorption was severely depressed (Sitrin and Bengoa, 1987). In the diet of chicks, $25(OH)D_3$ has been shown to have a 17% greater rate of absorption than vitamin D_3 (Bar, et al., 1980).

Vitamin D_3 metabolites are circulated within the body through binding with the vitamin D binding protein (DBP) in the blood (Norman and Hurwitz, 1993; Brown, et al., 1999). The binding affinities of the various vitamin D_3 metabolites to DBP differ, with $25(OH)_2 D_3 > 1, 25(OH)_2 D_3 >$ vitamin D_3 (Soares, et al., 1995). This DBP interacts with tissues and cells though the vitamin D receptor (VDR). VDR has been isolated from chick intestine, bone, kidney, parathyroid gland, pancreas, pituitary, chorioallantoic membrane and the egg shell gland (Norman, 1987). In addition to the classical tissues, in humans and rodent species the VDR has also been isolated from cells of the immune system (Manolagas, et al., 1985; Deluca and Cantorna, 2001; Griffin, et al., 2003). The presence of this VDR on immune cells in poultry has not been reported. It was the discovery of the VDR on cells of the immune system that led to the theory the vitamin D was involved in immune function regulation.

1.4.3. Vitamin D and Calcium Metabolism

Vitamin D_3 plays a major role in Ca metabolism and therefore is required for bone development and eggshell formation. Ca metabolism is under the control of three hormones, parathyroid hormone (PTH), $1,25(OH)_2D_3$ and calcitonin. The first two are involved in stimulating Ca resorption from bone and the intestinal tract when needed by the chicken (Norman and Hurwitz, 1993). Plasma PTH increases in response to low plasma Ca which stimulates bone resorption and aids in the kidney production of 1-hydroxylase which in turn stimulates the production of $1,25(OH)_2D_3$ (Norman and Hurwitz, 1993). $1,25(OH)D_3$ stimulates production of proteins (specifically calbindins) required for Ca uptake from the intestinal tract (Watkins, 1993; DeLuca, 2004) and is

involved in Ca resorption from the kidney (Yamamoto, et al., 1984). Under normal conditions in the chicken, Ca uptake from the gut is about 70% vitamin D-dependent (Hurwitz, et al., 1983). Vitamin D is therefore, crucial in Ca homeostasis so that Ca can be available for bone formation and other critical functions. The role of calcitonin in Ca homeostasis is still undefined in birds (Norman and Hurwitz, 1993), however in other species this hormone works to stop bone Ca resorption under hypercalcemic conditions (Combs, 1992).

1.4.4. Vitamin D and Poultry Production

1.4.4.1. Vitamin D requirement by the bird

Current recommended levels of vitamin D_3 are 200, 190 to 300 and 1100 ICU for the broiler chick, laying hen and turkey, respectively (National Research Council, 1994). The NRC does not list a required level of vitamin D for the broiler breeder, although a level of 3,000 ICU has been recommended for the Cobb broiler breeder (Cobb-Vantress Inc, 2005). The much higher recommended level of dietary vitamin D_3 by the Cobb breeder company represents the level needed for optimum performance of the breeder hen. For example the Cobb broiler management guide recommends 3,000 to 4,000 ICU of vitamin D for broilers (Cobb-Vantress Inc, 2004), compared to the 200 ICU recommended by the NRC (National Research Council, 1994). Recent research has also questioned the required levels of vitamin D for broilers. Whitehead et al. (2004) suggests that levels 7 to 10 times the NRC are needed for optimal cortical bone development up to 14 d and levels 50 times the NRC are needed to prevent tibial dyschondroplasia (TD) in broilers. It is worth noting as well that the required vitamin D_3 levels reported by

Whitehead et al. (2004) are defined at optimal dietary Ca and available P (aP) intakes. The explanation for the different requirement levels are that some requirements are assessed for some defined optimum performance (ie. breeder recommendations), while others are likely the amount that would simply prevent a deficient state (ie. NRC).

1.4.4.2. Vitamin D and Poultry Bone Development

The vitamin D metabolite, $1,25(OH)_2D_3$, is involved in the deposition of skeletal minerals as well as Ca resorption from the bone tissues when plasma Ca levels are low (DeLuca, 2004). $1,25(OH)_2D_3$ and 24, $25(OH)_2D_3$ are required for the proper development of the growth plate cells (chondrocytes) (Ornoy, et al., 1978). The VDR has been isolated from growth plate chondrocytes (Berry, et al., 1996).

The strength and quality of bone has been shown to be affected by dietary vitamin D source and level. Tibia weight, density and breaking strength all increased with increasing levels of $1,25\text{-}(OH)_2D_3$ from 0 to 1 μg/kg in the diet of 75 wk-old laying hens (Frost and Roland, 1991). Vitamin D at high levels (>1,000 ICU) can prevent TD in broilers (Luo and Huang, 1991; Whitehead, et al., 2004).

1.4.4.3. Vitamin D and Eggshell Quality

The role of vitamin D in Ca metabolism makes it an especially important nutrient for egg-laying poultry. Approximately 2 g of Ca goes into each egg (Roland and Farmer, 1984), which represents a significant Ca output by the hen for its entire egg production cycle (>300 eggs for table egg laying hens and >180 eggs for broiler breeders). Due to the different feeding strategies between table egg layers (fed ad libitum) and broiler

breeders (feed restricted), it is reasonable to assume that some aspects of Ca metabolism may also differ. Past research has shown that Leghorn-type hens will increase Ca consumption in response to a low plasma levels of Ca (Hughes and Wood-Gush, 1971; Roland, et al., 1973). As broiler breeders are feed-restricted and usually fed in the early morning, breeder hens are not able to increase Ca consumption if needed later in the day or night. When broiler breeders received most of their Ca in the morning, the amount of dietary Ca available for egg shell formation and egg shell was much lower than if the broiler breeders received Ca in the afternoon, when shell formation would likely be beginning (Farmer, et al., 1983). Shell quality is important in the broiler breeder industry as it significantly affects egg hatchability both of which are economically important factors for producers.

1.4.4.4. Vitamin D and Chick Embryonic Development

Vitamin D_3 status of the broiler breeder can significantly affect the development of the chick embryo and subsequently its hatchability (Wilson, 1997). The vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 contents within the egg yolk (Mattila, et al., 1999). The egg yolk, which is the main source of nutrition for the developing embryo and newly hatched chick (Speake, et al., 1998), has a specific DBP (Fraser and Emtage, 1976; White, 1987). The DBP has a high affinity for vitamin D_3 but also binds 25-OH D_3, and both are incorporated into the yolk (Edelstein, et al., 1973; Fraser and Emtage, 1976). When there is a high concentration of 25-OH D_3 in the hen's blood it can displace vitamin D_3 from the DBP such that more 25-OH D_3 than otherwise gets passed into the yolk (Fraser and Emtage, 1976).

Interestingly, the active vitamin D metabolite, $1,25(OH)_2D_3$ does not get passed from the hen into the egg yolk. When $1,25(OH)_2D_3$ is provided as the sole vitamin D_3 source to the hen, normal embryonic growth does not occur due to vitamin D deficiency in the egg (Henry and Norman, 1978; Sunde, et al., 1978; Soares, et al., 1979; Ameenuddin, et al., 1983; Hart, et al., 1986; Ameenuddin, et al., 1987). Chick embryos from hens fed no dietary vitamin D_3 or fed only $1,25(OH)_2$ D_3, have upper mandible abnormalities and usually die at 18 to 19 d of embryonic life (Sunde, et al., 1978).

The metabolism of vitamin D_3 is important within the developing embryo. The enzyme, 1α-hydroxylase, which is responsible for the hydroxylation of 25-OH D_3 to 1, $25(OH)_2D_3$ is present as early as d 12 of incubation and increases in concentration during further development (Turner, et al., 1987). Previous studies have shown that vitamin D_3 is hydroxylated to 25-OH D_3, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ within the chick embryos (Moriuchi and Deluca, 1974; Bishop and Norman, 1975; Kubota, et al., 1981). The early development of vitamin D metabolism within the chick signifies the importance of this nutrient to the growing embryo. Quail eggs deficient in vitamin D and therefore limited embryonic formation of $1,25(OH)_2$ D_3, do not hatch because Ca transport across the chorioallantoic membrane from the shell to the embryo is dependent on $1,25(OH)_2$ D_3 (Elaroussi and DeLuca, 1994; Elaroussi, et al., 1994b). However, the addition of 1,100 ICU of 25-OH D_3 to the diet of turkey breeders, that also included 2,200 ICU of vitamin D_3, improved egg hatchability as compared to dietary vitamin D_3 alone (Manley, et al., 1978).

1.4.4.5. Vitamin D, 25-OH D_3 and Broiler Production

Vitamin D is required for growth, health and bone development in the chick. In Canada, broiler production typically takes place in light-tight, environmentally controlled barn facilities. This impairs the UV-dependent synthesis of vitamin D that takes place in animals exposed to the sun (Norman and Hurwitz, 1993). Therefore a dietary source of vitamin D_3 is necessary for broiler production.

The most abundant circulating form of vitamin D is 25-OH D_3, plasma levels of which give a good indication of the vitamin D status of the chick (Haussler and Rasmussen, 1972). When either vitamin D_3 or 25-OH D_3 amounts are increased in the diet of the bird, the circulating level of 25-OH D_3 also increases (Yarger, et al., 1995b). However, feeding increasing doses of dietary 25-OH D_3 resulted in a more rapid rate of increase of plasma 25-OH D_3 than did vitamin D_3 diet at similar vitamin D_3 activity levels (Yarger, et al., 1995b).

The natural hepatic production of 25-OH D_3 can become impaired, either due to stresses such as infection or mycotoxin feed toxicity (Waldenstedt, 2006) or perhaps due to immaturity of enzyme development required for vitamin D absorption in the young chick (Ward, 2004). Therefore, the opportunity exists to improve the vitamin D status of the chick by feeding dietary 25-OH D_3. This product is safe for use in poultry and showed no signs of toxicity at up to 10 times the recommended feeding concentration of 69 µg/kg (Yarger, et al., 1995a). Providing the chick with a dietary source of 25-OH D_3 may allow it to be readily available for the conversion to $1,25(OH)_2D_3$ and therefore offer the potential to enhance the functions that vitamin D metabolites serve within the body. Currently, 25-OH D_3 is commercially available for use in poultry diets under the trade name HyD®. When comparing the bio-potency vitamin D_3 and 25-OH D_3, the

latter was found to be anywhere from 1 up to 4 times more potent than vitamin D_3 when looking at plasma Ca^{2+}, bone ash and strength (Haussler and Rasmussen, 1972; Boris, et al., 1977; McNaughton, et al., 1977; Cantor and Bacon, 1978; Soares, et al., 1978). Previous studies have shown dietary 25-OH D_3 to increase BW (Yarger, et al., 1995b; Mitchell, et al., 1997; Aburto, et al., 1998), improve feed conversion efficiency and to increase breast muscle yield in broilers (Yarger, et al., 1995b) in comparison with vitamin D_3.

1.4.4.6. Vitamin D and Innate Immune Function

Nutrition plays a major role in immune function and the overall health of the bird. Nutritional deficiencies often lead to immune function depression, making the bird more susceptible to diseases and infections (Kidd, et al., 2001). Vitamin D is involved in various aspects of the immune system. Reports on its involvement in the immune function of poultry species has been limited (Aslam, et al., 1998; Huff, et al., 2000; Huff, et al., 2002; Fritts, et al., 2004). However, human and rodent research has linked it to various aspects of both the acquired and innate immune function such as but not limited to; promoting monocyte maturation, neutrophil chemotaxis, enhancing phagocytic and bactericidal/tumorcidal capability of leukocytes, enhancing antigen presentation, inhibiting IL-2, suppressing inflammatory T cell response and inhibiting lymphocyte proliferation (Cohen and Gray, 1984; Gray and Cohen, 1985; Manolagas, et al., 1985; Reinhardt and Hustmyer, 1987; Manolagas, et al., 1989; Binder, et al., 1999; Brown, et al., 1999; Deluca and Cantorna, 2001; Griffin, et al., 2003; Cantorna, et al., 2004; Gomme and Bertolini, 2004; Gombart, et al., 2005).

In humans, one of the interesting aspects of vitamin D is its role in macrophage development and function. Vitamin D promotes the maturation of monocytes into macrophages (Manolagas, et al., 1985; Provvedini, et al., 1986). In the chick, vitamin D deficiency decreased the macrophage population (Aslam, et al., 1998). In addition, mouse macrophages require $1,25(OH)_2 D_3$ for activation (Gavison and Bar-Shavit, 1989). In the presence of $1,25(OH)_2 D_3$, the number of VDR on the mouse macrophage increases significantly (Veldman, et al., 2000), which most likely aids in the activation of the macrophage. Furthermore, human and bovine studies have shown that $1,25(OH)_2D_3$, induces macrophage nitric oxide production (Rockett, et al., 1998; Waters, et al., 2001), which is one of the major macrophage microbicidal mechanisms. The vitamin D metabolite, $1,25(OH)_2 D_3$, in humans also boosts phagocytic activity by enhancing the expression of the Fc receptors (Boltz-Nitulescu, et al., 1995). Another unique characteristic of macrophages is that they can produce $1,25(OH)_2 D_3$ from $25(OH)_2D_3$ when activated, as the macrophage has the 1α-hydroxylase enzyme (Mathieu and Adorini, 2002). The vitamin D metabolite, $1, 25(OH)_2D_3$ increases the survival of macrophages at the higher body temperatures that accompany an inflammatory immune response (Brown, et al., 1999). Additionally, the other major phagocytic cells, neutrophils, have increased chemotactic activity when bound with the DBP (Gc-globulin) (Binder, et al., 1999).

As the avian immune system begins to develops very early in ovo (Sharma, 1997), maternal dietary 25-OH D_3 supplementation may alter immunocompetence of the chick at hatch as it is passed from the hen into the egg yolk (Fraser and Emtage, 1976). As levels of 25-OH D_3 are not as tightly regulated within the body as its more potent

metabolite, $1,25(OH)_2D_3$ (Combs, 1992), the addition of 25-OH D_3 to the breeder diet may therefore offer a greater potential in improving immunocompetence of the chick. In addition, $1,25(OH)_2D_3$ is not passed from the hen into the egg which often results in low hatchability and deformed chicks at hatch (Henry and Norman, 1978; Sunde, et al., 1978; Soares, et al., 1979; Ameenuddin, et al., 1983; Hart, et al., 1986; Ameenuddin, et al., 1987). The abundant number of studies indicating a regulatory role for vitamin D and its metabolites within the immune system of various other species suggests the possibility of similar roles within the poultry species, although little research has been done to support this.

1.5. Research Application

Bone quality is an area of increasing research activity because of the welfare and economic implications for all poultry species. Skeletal lameness and breakage not only hinder bird productivity (Rath, et al., 2000) but also are assumed to cause pain to the bird (Whitehead and Fleming, 2000). Fast growth rates of broilers and turkeys and high egg production accompanied with light BW of laying hens have been implicated in several skeletal disorders (Lilburn, 1994; Fleming, et al., 1997; Whitehead and Fleming, 2000). As technology advances, new methodology for assessing bone quality arises. Quantitative CT has been used successfully in human bone biology but the recent introduction of it into poultry bone studies requires further development. This QCT technology would allow for more in-depth research into poultry bone biology with analysis of cortical and trabecular BMD and cross-sectional areas.

Vitamin D is involved in many functions within the bird from calcium absorption and bone development and maintenance to immune function. Vitamin D needs to be metabolized in the liver to 25-OH D_3 and then in the kidney to the active vitamin D metabolite, $1,25(OH)_2$ D_3. Currently, 25-OH D_3 is commercially available for use in poultry diets under the trade name HyD® (DSM Nutritional Products, Parsippany, NJ). Providing the chick with a dietary source of 25-OH D_3 may allow for a more readily available metabolite for the conversion to $1,25(OH)_2D_3$ and the potential to enhance the functions that vitamin D metabolites serve within the body.

Therefore, the overall goal of the research in the following chapters was to first evaluate QCT as a method of assessing bone quality and poultry that would then be used in conjunction with assessing both direct and maternal supplementation of 25-OH D_3 on broiler production, bone quality and immune function. This goal was achieved in the thesis research by testing the following hypotheses:

1. **It was hypothesized that QCT would provide a precise BMD measure of poultry bones.**

This hypothesis was addressed in Chapter 2 of the thesis and includes a secondary objective, where we examined the effect of bone handling treatments, which are commonly employed prior to BMD analysis, on QCT BMD and cross-sectional area.

2. **It was hypothesized that QCT BMD and cross-sectional area would correlate with the traditional methods of bone quality evaluation.**

This hypothesis was addressed in Chapter 3, by comparing QCT BMD and cross-sectional area to traditional methods of bone quality evaluation.

3. **It was hypothesized that dietary 25-OH D_3 would enhance broiler production traits, bone quality and the inflammatory immune response as compared to dietary vitamin D_3.**

This hypothesis was addressed in Chapter 4 in which the objectives of the study were to investigate the effects of dietary 25-OH D_3 (and age at receiving dietary 25-OH D_3) on broiler production traits, plasma 25-OH D_3, bone formation and quality and carcass composition at 6 wk of age. This hypothesis was further addressed in Chapter 5 in which the objectives were to examine the effects of dietary 25-OH D_3, alone or in combination with vitamin D_3 on broiler performance, bone quality, the inflammatory response in broilers.

4. **It was hypothesized that maternal dietary 25-OH D_3 would support normal broiler breeder production, improve BMD, hatchability, progeny production performance as well as lead to a more mature innate immune system of their progeny at hatch.**

This hypothesis was addressed in Chapter 6 where the objective of the research was to investigate the effects of maternal 25-OH D_3 on fertility, hatchability, chick quality, chick production traits, plasma 25-OH D_3, and bone quality. This hypothesis was further addressed in Chapter 7 where the objectives of the study were to investigate the effects of

maternal dietary 25-OH D_3 on broiler breeder production traits and BMD as well as *in vitro* innate immune function of the chicks.

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CHAPTER 2: Precision of Quantitative Computed Tomography as a Tool for Assessing Bone Quality in Poultry

2.1. INTRODUCTION

A sound skeletal system is required to enable birds to move about freely to reach feed and water necessary for growth and production in commercial poultry species. Skeletal lameness and breakage not only hinder bird productivity (Rath, et al., 2000) but also cause pain to the bird (Webster, 2004). Fast growth rates of broilers and turkeys, and high egg production, accompanied with lower BW of laying hens, have been implicated in several skeletal disorders (Lilburn, 1994; Fleming, et al., 1997; Whitehead and Fleming, 2000).

There are 2 main bone types in birds; cortical bone is the outer shell that provides most of the structural strength and trabecular bone, which is found within the endocortical space and also adds to the structural integrity of the bone (Whitehead and Fleming, 2000). In female birds a third type, medullary bone, forms on the surfaces of the trabecular bone spicules and endocortical surfaces as the hen nears sexual maturity (Whitehead, 2004). Medullary bone acts as a labile Ca source from which the hen can draw upon when dietary Ca is insufficient during eggshell formation (Hurwitz, 1965; Candlish, 1971). Unlike structural bone, the medullary bone can be replaced while the hen is in production (Whitehead, 2004).

Bone mineral density measured by Quantitative Computed Tomography (QCT) is currently used in the medical profession to monitor osteoporosis in humans in both research and clinical situations (Genant, et al., 1987; Wachter, et al., 2001). Quantitative

computed tomography calculates the true volumetric BMD based on multiple x-rays of a given bone section taken a many different angles, which provides a 3-dimenisional measurement of bone mineral density and distribution (Korver, et al., 2004). The main advantages of QCT are that it is a noninvasive measure of BMD and allows for the separate measurement of BMD and areas of the total, cortical and trabecular bone regions (Genant, et al., 1987). In humans, QCT has been shown to yield similar results as histomorphometry (Rosen, et al., 1995), dual energy radiography in healthy and osteoporotic subjects (Pacifici, et al., 1990), bone ash (Genant, et al., 1987; Waite, et al., 2000), bone Ca (Markel, et al., 1991), lateral and posteroanterior dual x-ray absorptiometry (Genant, et al., 1987; Guglielmi, et al., 1994) and spinal fracture index (Genant, et al., 1987). Some studies have found QCT to be superior to dual energy x-ray absorptiometry (Pacifici, et al., 1990; Guglielmi, et al., 1994; Rosen, et al., 1995) in predicting osteoporosis in humans.

In poultry studies, the use of QCT would allow for close monitoring of changes in BMD and cross-sectional area of the cortical and trabecular bone sections over time, that might not be apparent using measures such as total bone ash, Ca and strength measurements. QCT has only recently been introduced for use in poultry bone metabolism research (Korver, et al., 2004; Kim, et al., 2007). Therefore, the objective of this study was to evaluate QCT precision. We hypothesized that QCT would provide a precise BMD measure of poultry bones. As a secondary objective, we examined the effect of bone handling treatments that are commonly employed prior to BMD analysis, on QCT BMD and area.

2.2. MATERIALS AND METHODS

2.2.1. Bone Mineral Density and Area

Bone density and area analyses were performed using a Stratec Norland XCT quantitative computed tomography scanner with a 50kV x-ray tube (XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA) as the method described by Riczu et al (2004). Briefly, the scanner was calibrated daily using a multi-slice standard phantom (XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA). The phantom contained a series of materials of known densities to create a standard attenuation curve. For each bone, a longitudinal scout view was obtained to locate the proximal and distal ends of the bone. Based on these markers, the location on the bone for the cross-sectional x-ray density and area analysis were set. At that time, a 1 mm cross-sectional x-ray slice with a voxel size of 0.1 mm was set for the region of interest on each bone. Norland XMENU software version 5.40C was used to analyze the resulting cross-sectional total, cortical, and trabecular BMD and cross-sectional areas. Total BMD was the weighted average of both the cortical and trabecular bone, and reflected the amount and the density of each bone section. Cortical BMD was the outer shell of the bone that was determined to be >500 mg/cm^3. Bone mineral density within the trabecular region is bone in the trabecular space and it includes trabecular and medullary bone tissue (in studies involving egg-laying birds) to calculate the density of this region. Cross-sectional area of the trabecular bone region is calculated whether bone is present or an empty space. Soft tissue surrounding the bone was differentiated from the outer cortical bone surface, an inner threshold level was set at 400 mg/cm^3 to separate cortical and subcortical from trabecular bone and a threshold of 500 mg/cm^3 was used to separate cortical from sub-

cortical bone regions. These threshold values were reached after trying different thresholds to find which worked best and are consistent with what is used in the human literature (Cheng, et al., 1997; Lochmuller, et al., 2002).

2.2.2. QCT Precision Test

Six healthy broilers (42 d of age) and 8 healthy broiler breeders (65 wk of age) were selected at random from larger populations for study. The right shank (tarsometatarsus) was scanned 3 consecutive times (with complete removal of bird/bone between scans) at both the midpoint (50% of bone length) and at 60% of the length of the bone from the proximal end for all bone treatments. For the live scan, birds were anesthetized with an intramuscular injection of a combination of 20 mg/kg of BW of a 100 mg/ml solution of ketamine hydrochloride (Ketalean, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and 2 mg/kg of BW of a 20 mg/ml solution of xylazine (Rompun, Bayer Inc., Agriculture Division–Animal Health, Etobicoke, Ontario, Canada). Live scans were performed 3 times per bird with birds removed from the QCT unit and repositioned between each scan. The birds were killed by cervical dislocation after the third scan, prior to recovery from anesthesia. The right leg was then removed from each bird and 3 scans per tarsometatarsus were conducted as described above on each bone after being subjected to various handling treatments. After removal from the bird, each tibiotarsus was scanned (Fresh treatment). Each bone was then frozen for 1 d with the flesh on, thawed completely and scanned again with the flesh intact (FF treatment) and then with flesh removed (FF-SWF treatment). Following flesh removal and scanning, each bone was frozen again for 1 d. After thawing, the bones without soft

tissue (FWF treatment) were scanned again. The bones were then oven-dried for 24 h at 110 C and scanned once more (Dried treatment). For the 65 wk-old broiler breeder precision test, the FF-SWF treatment was not applied. Standard deviation and CV of the repeated measurements within each bird and bone treatment were calculated using the root mean square method to determine QCT precision (Glüer et al. 1995).

2.2.3. Statistical Analysis

Precision was expressed on a percentage basis by calculating the CV of the multiple scans performed on each individual broiler or broiler breeder bone within each handling treatment as given in the calculation below, and then taking the root mean square average of the multiple subjects (Gluer, et al., 1995). This allowed for the comparison of variation in the QCT scans within each treatment and across the various treatments.

$$CV_{SD} = \left(SD / \sum_{j=1}^{m} \overline{x_j} / m \right) \bullet 100$$

Where: SD = standard deviation; x_j was the mean of all measurements on an individual; m = number of measurements per individual; and j was the number of individuals (since each calculation was done on an individual basis and then averaged, j=1).

The Proc Means procedure of SAS was used to determine differences in BMD and cross-sectional area due to bone handling treatment (SAS Institute, 1999). Correlations of BMD and cross-sectional area measurements among the different bone handling treatments were assessed using the Proc Corr procedure of SAS (SAS Institute, 1999). The level of significance, unless otherwise stated, was assessed at a probability of $P \leq 0.05$.

2.3. RESULTS AND DISCUSSION

2.3.1. Bone Handling Treatment Effects on Bone Mineral Density and Cross-sectional Area.

Broiler cortical BMD at the midpoint was not affected by the different bone treatments; however at 60% distal length BMD was greater in the FF-SWF treatment than the previous treatments. The Dried bones had the greatest cortical BMD at the 60% distal scan location (P=0.0001, Table 2.1). Previously, drying bones has been shown to increase bone breaking strength (Orban, et al., 1993). An increase in cortical BMD would contribute to increased bone breaking strength as it is the cortical bone that provides the majority of the intrinsic bone strength.

Increasing the amount of handling resulted in decreasing trabecular BMD (P<0.05; Table 2.1). Bones from the FF-SWF had reduced trabecular BMD at both the midpoint and 60% distal scan locations relative to the previous treatments. Furthermore, bones in the Dried group had no measurable trabecular BMD in any bone sample and therefore a CV could not be calculated for this treatment (Table 2.1). The trabecular BMD tended to decrease after each bone handling treatment, and the precision error increased among the bones. It is believed because the QCT scans were performed in areas of the bone that do not typically contain trabecular bone that the trabecular BMD reading from the QCT unit is the density of the material (i.e. bone marrow) in that defined space. This would explain the "disappearance" of the trabecular BMD when dried as bone marrow would contain a substantial amount of moisture that would be lost during the drying procedure. Therefore, the previous measures prior to drying that included marrow within the trabecular space in the trabecular reading could have been altered by the disappearance of the marrow.

Broiler total BMD at the midpoint and 60% distal length scans tended to decrease with increased handling (P=0.02 and 0.06, respectively, Table 2.1). The FWF and Dried treatment groups had decreased overall total BMD for the midpoint measures relative to the live and fresh bone scans, primarily due to the apparent loss of trabecular bone ($P < 0.05$; Table 2.1).

Increased bone handling of the broiler tarsometatarsi resulted in a trend towards a reduction in cortical cross-sectional area at both the midpoint ($P<0.06$) and 60% distal ($P<0.06$, Table 2.1) scan locations. There was no effect of bone handling treatment on trabecular cross-sectional area. The Dried treatment group had a decreased total bone area for both the mid-shaft (relative to the Live and Fresh measurements, $P<0.03$) and 60% distal length (relative to the Live, Fresh and FF measurements, $P<0.003$, Table 2.1). Bone cross-sectional area was not expected to be affected by the different bone handling treatments as it is more of a static measurement than BMD. It is possible that a slight change in the location of the scan would alter the resulting bone area measurement. However, at the scan locations chosen along the bone shaft the areas surrounding the midpoint and 60% distal locations on the bone are fairly consistent in bone shape and size. Therefore, a slight shift to the left or right of the previous scan would not affect the area measurement to a significant extent. In addition, it is very easy to select the same scan location on an excised bone as the scout scan performed prior to the BMD scan gives exact length of each bone and makes it easy to select the desired location. It is possible that the moisture content within the bone, which may be lost during handling procedures, could result in a change in the bone cross-sectional area, although to date there is no literature to support this.

The broiler breeder cortical BMD measurement at the midpoint increased from the Live to Fresh treatments ($P<0.0001$, Table 2.2); this effect was not observed for the 60% distal length scan. However, cortical BMD at both the midpoint and 60% distal length increased from FF to FWF and increased further in the Dried treatment group ($P<0.05$; Table 2.5 and 2.6). The midpoint trabecular BMD increased from the Live to Fresh treatments (Table 2.2), whereas there was a decrease from FF to FWF and a further decrease when bones were dried ($P<0.0001$; Table 2.2). There was no measurable trabecular BMD at the midpoint after drying (Table 2.2). At the 60% distal location, there were sequential decreases in trabecular BMD from the FF to FWF and then to the Dried treatment ($P<0.0001$). Again, as discussed with the broiler bone handling, it is not certain that the trabecular BMD measurement was a reading of the density of material in that defined space, which may not necessarily be trabecular bone. This material, like bone marrow, would be altered by the bone handling due to the moisture content and the effects on that during the freezing, thawing and drying procedures.

Broiler breeder Live measures of cortical, trabecular and total cross-sectional area at the mid-shaft were greater than in the other bone treatment groups ($P<0.004$, Table 2.2). Repeated freezing and thawing tended to gradually decrease the bone cross-sectional area measurements. In the 60% distal scans, total and cortical areas increased from live to fresh measurements ($P<0.007$; Table 2.6). However, there were no consistent patterns among bone treatments for the differences in bone area, and even opposite effects occurring in the cortical bone area for the midpoint and 60% distal length scans. It is unclear as to why bone area was affected by bone handling treatments, although the explanation for these changes would be similar to those discussed for the broiler bones.

In the current study, the bone handling methods had a cumulative effect on the bones. However, the effects of repeated freezing and thawing as well as the bone preparation method did not affect each of the BMD or area measures in a consistent direction. For the broilers, BMD measured in the Live, Fresh and FF bones, for the most part were all similar for both the midpoint and 60% scan, however following further treatment, BMD and area begun to decrease with subsequent bone handling. For the broiler breeder bones, bone handling treatments had a greater effect on BMD and area measures than on the broilers. Although total BMD at both the midpoint and 60% scans remained unchanged for the broiler breeder bones throughout the different bone handling treatments, most other measures were greatly changed after each bone handling treatment. It was expected that bone handling treatments would have a minimal effect on QCT BMD measurements as BMD and area should be a fairly stable measure in excised bones. Many of the results were not expected and have not been reported before; thereby making explanations as to why they occurred would simply be speculations. However, as this experiment was designed to assess the precision and use of QCT in determining poultry BMD and bone area more consideration was given to what happen to the measure than what happened to bone after storage treatments. In recognition of the changes in the BMD and area measures it is recommended that similar bone handling treatment as well as scanning position be used within an individual study to maintain consistency within the results. Repeated freezing and thawing may reduce the quality of the data collected.

2.3.2. Precision of QCT Measurements.

Cortical and total BMD measurements of the broiler tarsometatarsi at the midpoint throughout all bone handling treatments were very repeatable. The average CV was between 0.62 and 1.13% for cortical density, and between 1.73% and 4.28% for total density (Table 2.1). Broiler tarsometatarsi cortical and total BMD measured at the 60% distal length for all the treatments had CV ranging from 0.88 to 3.48% and 0.37 to 1.46%, respectively (Table 2.1). Trabecular BMD of broiler tarsometatarsi were more variable within each bone treatment, with CV ranging from 6.72 to 35.88% and 4.99 to 14.79%, for the midpoint and 60% distal length, respectively (Table 2.1). The CV for this measure also tended to increase with increased bone handling (ie. more freezing and thawing).

Cortical, trabecular and total cross-sectional area measurements of broiler tarsometatarsi were less variable than BMD measurements and produced more consistent and precise results. The CV of the means ranged from 0.17 to 5.72 within all bone treatments for the broilers at both the mid-shaft and 60% distal length measurements (Table 2.1). Bone cross-sectional area was less affected by bone handling procedures than BMD as the area is a measure on the size of the bone or bone fractions, which is unlikely to change when repeatedly frozen and thawed. This is not the case with BMD, which appeared to be affected to a greater extent after bone handling procedures as this measure would be more sensitive to freezing and thawing, as well as small changes in positioning of the bone.

Similar to the broiler results, cortical and total BMD at the mid-shaft and at 60% distal length of broiler breeder tarsometatarsi were very repeatable within an individual bird (CV ranging from 0.5 to 3.8% and 0.7 to 3.1%, respectively; Table 2.2). The 65 wk-

old broiler breeder trabecular BMD measurements were more variable than other BMD measures within the bones, having CV ranging from 6.6 to 13.9 and 8.9 to 16.1% for the midpoint and 60% distal length measurements, respectively (Table 2.2). The cortical, trabecular and total cross-sectional area measurements of broiler breeders were less variable than BMD measurements and produced more consistent results. The CV of the cross-sectional area means ranged from 0.4 to 6.9% within all bone treatments for the broiler breeders at both the mid-shaft and 60% distal length measurements (Table 2.2).

The BMD results in the current study for within-bird and within-treatment CV ranged from 0.37 to 35.88 for BMD and from 0.17 to 5.72 for bone cross-sectional area measurements (Tables 2.1 and 2.2). For the most part, the precision was <4% for BMD and <3% for bone cross-sectional area measurements. Trabecular BMD was more variable than cortical or total BMD. As the bone was stored (frozen and thawed repeatedly), trabecular BMD readings gradually decreased and eventually was often not measurable after drying. In this case, the reading the QCT unit gave for trabecular bone was likely a density reading of the marrow within the trabecular space. The midpoint, as well as the 60% distal length, region are areas of the bone that would have little to no trabecular bone, as this type of bone is mainly concentrated at the bone ends (R.H. Fleming, Roslin Institute, Roslin, Midlothian, UK, Personal communication). This would explain the effects of the cumulative bone treatments on the trabecular BMD readings, as the marrow density would likely be altered as the bone is repeatedly frozen and thawed and eventually dried as it is contains substantial amounts of adipose tissue (Blebea, et al., 2007). It was interesting that the effect of bone handling treatment was similar for both broiler and broiler breeder bones with respect to trabecular BMD. The broiler breeder

bones should have had medullary bone present throughout the entire bone at 65 wk of age. Therefore, either medullary bone has a very low density or the way that the QCT unit measures bone in the trabecular space makes it difficult to pick up on the diffuse nature of medullary bone.

The present precision results were similar and in some cases more precise than those reported using dual energy x-ray absorptiometry (DEXA) in horses at several different regions of interest along the equine metacarpus. In that study, CV ranging from 0.05 to 5.54 were reported when scanning each region 10 times (McClure, et al., 2001). More recently, BMD of the horse third metacarpal bone as measured by quantitative ultrasound and DEXA had very low CV (ranging from 0.65-0.92%) for the ultrasound and a slightly greater than 2% CV for the DEXA (Carstanjen, et al., 2003). However, that study utilized only one horse for the precision assessment, unlike the current study in which between 5 and 8 subjects were used. Precision in the current study may have been better if more scans had been performed per bone, however due to the necessity to anesthetize the live birds, only 3 scans were possible while the birds were unconscious. Swennen et al (2004), reported an average CV of 1.21 when using DEXA for measuring whole body BMD in chickens after 4 scans. DEXA provides a 2-dimensional radiographic density of the entire body or bone of interest (Grier, et al., 1996), whereas QCT provides a detailed 3-dimensional measure of a particular region of interest (Korver, et al., 2004). QCT offers many advantages to researchers in that it can provide a true volumetric BMD (Kalender, 2000). In addition, QCT also measures the different bone fractions (cortical and trabecular). Of these the cortical fraction is probably the most significant in poultry research as an indicator of bone strength and structure. The results of the current study

indicate that QCT allows for precise measurements of BMD and cross-sectional area in poultry. The trabecular BMD measure not only includes the density of the trabecular and medullary bone (if present), it is also influenced by the bone marrow density and therefore interpretation of such data needs to be addressed accordingly. However, the influence of trabecular BMD versus cortical BMD from a functional standpoint may be less important to the bird.

2.3.3. Correlations of QCT Measures between Various Bone Handling Treatments

Broiler cortical BMD at the midpoint was only correlated between the Live and Fresh, FF and Dried, FF-SWF and FWF, and FF-SWF and dried measurements for the 42 d old broiler tarsometatarsus ($r = 0.89$, 0.95, 0.84 and 0.90, respectively, $P<0.05$; Table 2.3). At the 60% distal length, Live cortical BMD was only significantly correlated with the Fresh cortical BMD ($r = 0.92$, $P<0.01$; Table 2.4). However, the FF-SWF, FWF and Dried cortical BMD were correlated with each other for the 60% distal length scans ($r =$ 0.98 to 0.99, $P<0.01$; Table 2.4). Trabecular BMD at the midpoint in Live birds was positively correlated with those of birds in the Fresh, FF, and FF-SWF treatments ($r =$ 0.86, 0.83 and 0.81, respectively, $P<0.05$; Table 2.3). In addition, midpoint trabecular BMD in Fresh bones were positively correlated with those of the FF-SWF and negatively related to the FWF treatments ($r=0.82$ and 0.90, respectively, $P<0.05$; Table 2.3). Trabecular BMD at the midpoint of the FF bones were positively correlated with those of the FF-SWF bones ($r=0.92$, $P<0.01$; Table 2.3). At the 60% distal length, trabecular mid-shaft BMD of the FF bones were correlated with the Live, Fresh and FF-SWF bones ($r =$ 0.88, 0.95 and 0.83, respectively, $P<0.05$; Table 2.4). The lack of measurable amounts of

trabecular bone in the Dried bone treatment did not make it possible to compare with the other bone handling treatments. Within individual bones, total BMD measurements at both the midpoint and at the 60% distal length were highly correlated among all bone handling treatments ($r = 0.82$ to 0.99, $P<0.05$ and $r = 0.94$ to 0.99, $P<0.01$, respectively; Tables 2.3 and 2.4).

Within individual bone samples, broiler bone area measurements from each of the bone treatments were all highly correlated for the broiler tarsometatarsus mid-shaft and 60% distal length measurements ($r = 0.89$ to 0.99, $P<0.05$ and $r=0.94$ to 0.99, $P<0.01$; Tables 2.3 and 2.4).

Broiler breeder Live cortical BMD for both the mid-shaft and 60% distal length scans were not correlated with any of the other cortical BMD from the other treatments (Tables 2.5 and 2.6). This shows that for cortical BMD, the live measurements can not be compared with any of the stored treatments. However, for both the mid-shaft and 60% distal length, the cortical BMD was correlated among the Fresh and FWF and Dried treatments ($r>0.83$, $P<0.01$ and $r>0.84$, $P<0.05$, respectively; Tables 2.5 and 2.6). At the mid-shaft, the Live measure of trabecular BMD was not correlated to the other bone treatments, however, positive correlations were found for the Fresh and FF ($r=0.88$, $P<0.01$) and FF and FWF ($r=0.88$, $P<0.01$; Table 2.5). Trabecular BMD at the 60% distal length scan of the Fresh bones was only correlated with the FF treatments ($r = 0.78$, $P<0.05$; Table 2.6). Within individual broiler breeder bone samples, total BMD was highly correlated among all bone treatments for both the mid-shaft and 60% distal length scans ($r = 0.69$ to 0.99, $P<0.05$; Tables 2.5 and 2.6).

Broiler breeder Live cross-sectional area measurements at the mid-shaft and 60% distal length of broiler breeder tarsometatarsi (cortical, trabecular or total) were not correlated with any of the area measurements from the other bone handling treatments (Tables 2.7 and 2.8). All other bone handling treatments were positively correlated with each other for, cortical and trabecular total bone area measurements at the mid-shaft scan ($r=0.79$ to 1.0, $P<0.05$; Table 2.5). Fresh, FF, FWF and Dried bone cortical areas were correlated at the 60% distal length scans ($r=0.96$ to 0.99, $P<0.0001$; Table 2.6). Fresh broiler breeder trabecular areas at the 60% distal length scans were correlated with the FWF and Dried areas ($r=0.99$, $P<0.0001$; Table 2.6); the FWF trabecular area was correlated with Dried trabecular area ($r=1.00$, $P<0.0001$; Table 2.6)

QCT measurements following the various bone treatments within an individual bird were all significantly correlated for total BMD and area for the broiler bones and total BMD for the broiler breeder bones at both the midpoint and 60% distal bone measurements. This suggests that total BMD and area of broiler bones as well as total BMD of broiler breeder bones measured after the bone is excised can be related to the measured counterpart of the live bird. However, within an individual bone, cortical as well as trabecular BMD were not correlated among most bone treatments for either the broiler or broiler breeder bones. It is suspected that the repeated freezing and thawing for the various bone handling procedures caused changes in these BMD, however, this effect may not be equal among each bone resulting in the low to no correlations for the different bone treatments.

2.4. CONCLUSIONS

BMD and area measurements were affected by freezing, thawing and drying treatments for both the broiler and broiler breeder bones. Therefore, it is important that bones be handled the same way within an experiment to allow valid comparisons to be made. In addition, the location of the QCT scan was also shown to be important, as the different bone handling treatments had different effects for the mid-shaft and 60% scans of the broiler breeders. Therefore, consistency with regards to the scan location and the bone handling treatment within an experiment are very important. In the current study the effects of the different handling treatments were cumulative as treatments were applied in a sequence, so prior handling treatments may have had an effect on the subsequent bone treatment. In conclusion, the results show that repeated freezing and thawing significantly affected the BMD therefore, if storing of the bones is necessary prior to BMD analysis it is recommended that the bones be frozen and thawed only once before analysis. In addition, most of the *ex vivo* density measures were not correlated with those of the live and therefore may necessitate the need to compare live measures with only other live measures. However, area was found to be correlated among all bone treatments of the broiler bones and therefore valid live to excised measures could be made. It is not known at this time why excised bone QCT measures would not be related to live measures of that same bone and therefore further investigation is required to determine if live QCT measures can be compared to QCT measures of same excised bone.

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Table 2.1 Precision of QCT measurements of 42-d-old broiler tarsometatarsi scanned at the midpoint and at 60% distal length following different bone handling treatments.

		Bone Treatment[1]						
		Live	Fresh	FF	FF – SWF	FWF	Dried	ANOVA
	n	6	6	6	6	6	5	P-value
		----------Tibiotarsus midpoint scan----------						
		BMD[5] (mg/cm^3)						
Cortical[6]	Mean	731.37	736.06	743.46	768.62	772.55	823.97	0.8064
	CV	1.11	1.05	1.13	0.72	0.62	0.82	
Trabecular[7]	Mean	85.07^{b}	97.69^{a}	90.76^{ab}	66.08^{c}	26.14^{d}	0	<0.0001
	CV	6.72	8.61	7.62	15.57	35.88	NA	
Total[8]	Mean	311.80^{ab}	315.99^{a}	307.85^{ab}	299.02^{ab}	268.86^{b}	237.41^{b}	0.0178
	CV	2.32	3.87	1.73	4.28	3.33	2.04	
		Area (mm^2)						
Cortical	Mean	16.70	16.56	16.48	15.18	14.68	13.18	0.0596
	CV	2.06	3.60	2.20	2.00	1.62	0.92	
Trabecular	Mean	33.48	34.02	34.98	32.16	32.25	29.04	0.7214
	CV	2.89	5.51	3.23	3.05	1.36	0.77	
Total	Mean	56.08^{a}	55.76^{a}	56.21^{ab}	49.55^{ab}	49.04^{ab}	43.71^{b}	0.0250
	CV	1.65	2.32	1.78	1.36	0.70	0.49	
		----------Tibiotarsus 60% distal scan----------						
		BMD (mg/cm^3)						
Cortical	Mean	751.06^{c}	744.76^{c}	762.78^{c}	798.93^{b}	802.22^{b}	851.89^{a}	<0.0001
	CV	1.46	0.94	1.30	0.92	0.37	0.57	
Trabecular	Mean	103.06^{a}	116.79^{a}	110.76^{a}	75.00^{b}	29.28^{b}	0	<0.0001
	CV	4.99	6.98	6.06	14.79	13.19	NA	
Total	Mean	358.67	356.83	353.78	343.46	312.42	304.57	0.0580
	CV	1.72	3.48	2.10	3.27	0.88	1.01	
		Area (mm^2)						
Cortical	Mean	17.46	17.18	16.78	15.17	14.89	13.52	0.0952
	CV	1.79	2.51	2.12	1.42	0.86	0.65	
Trabecular	Mean	27.71	28.42	28.97	26.33	26.40	24.01	0.3945
	CV	2.59	5.72	4.17	1.55	0.72	0.50	
Total	Mean	50.60^{a}	50.84^{a}	50.31^{a}	43.46^{b}	43.00^{b}	38.73^{b}	0.0029
	CV	1.10	2.94	2.62	0.93	0.57	0.17	

[1]The right tarsometatarsus of 6, 42 d-old broilers were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at the mid point of the bone. Scans were conducted sequentially on the same bones from live birds (Live), immediately following euthanasia on excised tibiotarsus (Fresh), after freezing with the flesh on and then thawed (FF), after freezing with flesh on, then thawed and scanned with flesh removed (FF-SWF), after freezing without the flesh and thawing (FWF) and after drying at 110 C for 24 h (Dried).

[5]Bone mineral density; [6]Cortical = measurements taken on the area define as >500 mg/cm^3 and the outer part of the bone; [7]Trabecular = measurements taken in the inner part of the bone in the trabecular space; [8]Total = the total for the entire bone.

[a-d]Means within the same row with no common superscripts are significantly different (P<0.05).

Table 2.2. Precision of QCT measurements of 65 wk broiler breeder tarsometatarsus scanned at midpoint and a 60% distal length following different bone handling treatments[1].

		Bone Treatment[4]					
		Live	Fresh	FF	FWF	Dried	ANOVA
	n	8	8	8	8	8	P-value
		----------Tibiotarsus midpoint scan----------					
		BMD[5] (mg/cm^3)					
Cortical[6]	Mean	901.06^{d}	947.62^{c}	957.58^{c}	1038.00^{b}	1123.00^{a}	<0.0001
	CV	3.8	3.3	2.3	0.5	1.0	
Trabecular[7]	Mean	73.32^{b}	94.21^{a}	92.96^{a}	46.72^{c}	0.00	<0.0001
	CV	10.4	13.9	10.4	6.6	NA	
Total[8]	Mean	407.62	425.38	438.42	448.40	416.76	0.4964
	CV	1.6	1.7	1.3	1.0	1.0	
		Area (mm^2)					
Cortical	Mean	25.97^{a}	24.11^{b}	23.24^{bc}	22.43^{c}	20.84^{d}	<0.0001
	CV	3.7	1.9	0.9	0.5	0.4	
Trabecular	Mean	38.85^{a}	29.18^{b}	27.15^{b}	25.85^{b}	24.72^{b}	0.0036
	CV	3.8	2.7	1.2	0.9	0.8	
Total	Mean	68.47^{a}	56.83^{b}	52.53^{bc}	48.06^{c}	45.22^{c}	<0.0001
	CV	3.9	2.7	1.1	0.5	0.4	
		----------Tibiotarsus 60% distal scan----------					
		BMD (mg/cm^3)					
Cortical	Mean	986.10^{c}	972.37^{c}	981.11^{c}	1063.00^{b}	1115.00^{a}	<0.0001
	CV	1.4	2.3	1.5	0.9	0.7	
Trabecular	Mean	68.11^{a}	77.42^{a}	79.64^{a}	46.44^{b}	17.72^{c}	<0.0001
	CV	14.2	16.1	8.9	13.3	NA	
Total	Mean	501.34	507.48	523.92	548.81	529.87	0.4321
	CV	3.1	2.9	1.4	0.7	1.3	
		Area (mm^2)					
Cortical	Mean	22.89^{bc}	24.80^{a}	24.46^{ab}	23.62^{ab}	21.90^{c}	0.0067
	CV	2.4	1.1	0.9	0.8	0.4	
Trabecular	Mean	25.79	34.67	426.99	32.36	30.88	0.0574
	CV	6.9	2.0	2.1	0.9	0.5	
Total	Mean	50.83^{c}	62.53^{a}	59.44^{ab}	55.88^{abc}	52.69^{bc}	0.0186
	CV	4.9	2.1	1.1	0.9	0.4	

[1]The right tarsometatarsus of 6, broiler breeders were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at the mid point of the bone. Scans were conducted sequentially on the same bones from live birds (Live), immediately following euthanasia on excised tibiotarsus (Fresh), after freezing with the flesh on and then thawed (FF), after freezing without the flesh and thawing (FWF) and after drying at 110 C for 24 h (Dried).

[5]Bone mineral density.

[6]Cortical = measurements taken on the area define as >500 mg/cm^3 and the outer part of the bone.

[7]Trabecular = measurements taken in the inner part of the bone in the trabecular space; [8]Total = the total for the entire bone.

[a-d]Means within the same row with no common superscripts are significantly different (P<0.05).

Table 2.3. Correlations of QCT measurements of 42-d broiler tarsometatarsi scanned at the midpoint under different bone handling treatments[1].

	Live	Fresh	FF	FF-SWF	FWF
			Cortical[4] Density		
Fresh	0.89*				
FF	0.37	0.64			
FF-SWF	0.69	0.61	0.66		
FWF	0.54	0.23	0.20	0.84*	
Dried	0.40	0.43	0.95**	0.90*	0.77
			Trabecular[5] Density		
Fresh	0.86*				
FF	0.83*	0.69			
FF-SWF	0.81*	0.82*	0.92**		
FWF	-0.79	-0.90**	-0.52	-0.74	
Dried	N/A	N/A	N/A	N/A	N/A
			Total[6] Density		
Fresh	0.95**				
FF	0.99***	0.95**			
FF-SWF	0.99***	0.93**	0.97***		
FWF	0.90**	0.82*	0.90**	0.93**	
Dried	0.98**	0.92*	0.99***	0.99***	0.94**
			Cortical Area		
Fresh	0.98***				
FF	0.99***	0.99***			
FF-SWF	0.98***	0.96**	0.98***		
FWF	0.98***	0.96**	0.98***	0.99***	
Dried	0.99***	0.97**	0.99***	0.99***	0.99***
			Trabecular Area		
Fresh	0.91**				
FF	0.98***	0.93**			
FF-SWF	0.99***	0.93**	0.98***		
FWF	0.99***	0.89**	0.98***	0.99***	
Dried	0.98**	0.89*	0.99***	0.96**	0.99***
			Total Area		
Fresh	0.95**				
FF	0.99***	0.94**			
FF-SWF	0.99***	0.96**	0.99***		
FWF	0.99***	0.94**	0.99***	0.99***	
Dried	0.98**	0.95**	0.99***	0.99***	0.99***

[1]The right tarsometatarsus of 6, 42 d broilers were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at the mid point of the bone. [1]The right tarsometatarsus of 6, 42 d-old broilers were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at the mid point of the bone. Bone treatments for each individual bone included (in sequential order): Live, Fresh, Frozen with Flesh (FF), Frozen with Flesh on and scanned with flesh removed (FF-SWF), Frozen without Flesh (FWF), and after drying at 110 C for 24 h (Dried).

[4]Cortical = measurements taken on the area define as >500mg/cm^3 and the outer part of the bone.

[5]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[6]Total = the total for the entire bone.

*=P<0.05; **=P<0.01; ***=P<0.001.

Table 2.4. Correlation of QCT measurements at 60% distal length on 42-d broiler tarsometatarsi under different bone handling treatments[1].

	Live	Fresh	FF	FF-SWF	FWF
			Cortical[4] Density		
Fresh	0.92**				
FF	0.34	0.29			
FF-SWF	0.77	0.54	0.60		
FWF	0.70	0.48	0.61	0.99***	
Dried	0.75	0.65	0.64	0.98**	0.99***
			Trabecular[5] Density		
Fresh	0.70				
FF	0.88*	0.95**			
FF-SWF	0.74	0.77	0.83*		
FWF	0.30	0.34	0.35	0.68	
Dried	N/A	N/A	N/A	-0.47	N/A
			Total[6] Density		
Fresh	0.96**				
FF	0.97***	0.98***			
FF-SWF	0.99***	0.96**	0.96**		
FWF	0.98***	0.95**	0.94**	0.99***	
Dried	0.98**	0.98**	0.98**	0.96**	0.95**
			Cortical Area		
Fresh	0.99***				
FF	0.99***	0.98***			
FF-SWF	0.99***	0.97***	0.98***		
FWF	0.98***	0.96**	0.97***	0.99***	
Dried	0.99**	0.99***	0.97**	0.99***	0.99***
			Trabecular Area		
Fresh	0.96**				
FF	0.98***	0.94**			
FF-SWF	0.99***	0.96**	0.98***		
FWF	0.99***	0.94**	0.98***	0.99***	
Dried	0.99***	0.98**	0.98**	0.99***	0.99***
			Total Area		
Fresh	0.99***				
FF	0.99***	0.99***			
FF-SWF	0.99***	0.99***	0.99***		
FWF	0.99***	0.99***	0.99***	0.99***	
Dried	0.99***	0.99***	0.99***	0.99***	0.99***

[1] The right tarsometatarsus of 6, 42 d broilers were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at 60% of the distal length of the bone. Bone treatments for each individual bone included (in sequential order): Live, Fresh, Frozen with Flesh (FF), Frozen with Flesh on and scanned with flesh removed (FF-SWF), Frozen without Flesh (FWF), and after drying at 110 C for 24 h (Dried).

[4]Cortical = measurements taken on the area define as >500mg/cm^3 and the outer part of the bone.

[5]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[6]Total = the total for the entire bone.

*=P<0.05; **=P<0.01; ***=P<0.001.

Table 2.5. Correlations of QCT bone density and area measurements of 65 wk broiler breeder tarsometatarsi scanned at the midpoint under different bone treatments[1].

	Live	Fresh	FF	FWF
		Cortical[4] Density		
Fresh	-0.03			
FF	0.08	0.21		
FWF	0.30	0.92***	0.10	
Dried	0.30	0.84**	0.05	0.92***
		Trabecular[5] Density		
Fresh	0.07			
FF	-0.34	0.88**		
FWF	-0.36	0.22	0.88**	
Dried	0.25	-0.34	0.44	0.31
		Total[6] Density		
Fresh	0.95***			
FF	0.95***	0.99***		
FWF	0.90**	0.96***	0.96***	
Dried	0.89**	0.90**	0.93**	0.92**
		Cortical Area		
Fresh	-0.17			
FF	-0.24	0.87**		
FWF	-0.27	0.79*	0.97***	
Dried	-0.25	0.85**	0.98***	0.99***
		Trabecular Area		
Fresh	0.07			
FF	0.19	0.98***		
FWF	0.13	0.97***	0.99***	
Dried	0.18	0.96***	0.99***	0.99***
		Total Area		
Fresh	-0.01			
FF	0.10	0.97***		
FWF	0.08	0.97***	0.99***	
Dried	0.13	0.97***	0.99***	0.99***

[1]The right tarsometatarsus of 6, broiler breeders were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at the mid point of the bone. Bone treatments for each individual bone included (in sequential order): Live, Fresh, Frozen with Flesh (FF), Frozen without Flesh (FWF), and after drying at 110 C for 24 h (Dried).

[4]Cortical = measurements taken on the area define as >500mg/cm^3 and the outer part of the bone.

[5]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[6]Total = the total for the entire bone.

*=P<0.05; **=P<0.01; ***=P<0.001.

Table 2.6. Correlations of QCT bone density and area measurements of 65 wk broiler breeder tarsometatarsi scanned at 60% distal length under different bone treatments[1].

	Live	Fresh	FF[2]	FWF[3]
		Cortical[5] Density		
Fresh	0.41			
FF	0.53	0.38		
FWF	0.54	0.82*	0.39	
Dried	0.32	0.84**	0.14	0.94***
		Trabecular[6] Density		
Fresh	-0.20			
FF	-0.54	0.78*		
FWF	-0.19	0.02	-0.08	
Dried	-0.66	0.13	0.06	0.21
		Total[4] Density		
Fresh	0.84**			
FF	0.85**	0.94***		
FWF	0.94***	0.94***	0.96***	
Dried	0.86**	0.69*	0.85**	0.82*
		Cortical Area		
Fresh	-0.23			
FF	-0.61	0.98***		
FWF	-0.18	0.96***	0.99***	
Dried	-0.18	0.98***	0.98***	0.99***
		Trabecular Area		
Fresh	-0.01			
FF	-0.01	-0.06		
FWF	-0.03	0.99***	-0.04	
Dried	0.01	0.99***	-0.04	0.99***
		Total Area		
Fresh	0.06			
FF	0.01	0.99***		
FWF	-0.01	0.99***	0.99***	
Dried	0.06	0.99***	0.99***	0.99***

[1]The right tarsometatarsus of 6, 65 wk broiler breeders were scanned using QCT, 3 times per bird for each bone treatment. Scans were preformed at 70% of the distal length of the bone. Bone treatments for each individual bone included (in sequential order): Live, Fresh, Frozen with Flesh (FF), Frozen without Flesh (FWF), and after drying at 110 C for 24 h (Dried).

[4]Cortical = measurements taken on the area define as >500mg/cm^3 and the outer part of the bone.

[5]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[6]Total = the total for the entire bone.

*=P<0.05; **=P<0.01; ***=P<0.001.

CHAPTER 3: Validation of Quantitative Computed Tomography as a Tool for Assessing Bone Quality in Poultry

3.1. INTRODUCTION

Several methods have commonly been utilized to determine bone characteristics in poultry. Bone breaking strength (Cheng and Coon, 1990; Elaroussi, et al., 1994), ash (Clunies, et al., 1992; Cransberg, et al., 2001; Hall, et al., 2003) and Ca content (Clunies, et al., 1992; Cransberg, et al., 2001; Petruk and Korver, 2004) are among the more commonly used methods of determining bone quality in poultry. These methods provide useful information for the study of bone quality but are limited in the information they provide. First, each of these procedures examines the whole bone and is not suitable for drawing conclusions on metabolically distinct bone fractions such as cortical, trabecular and medullary bone tissues, each of which can have distinct functions. Secondly, these procedures are necessarily performed *ex vivo*, therefore measurement of bone quality throughout the life cycle requires large numbers of birds to be killed at various times for bone analysis.

There are now new technologies available to assess bone mineral density (BMD) that will provide a more in-depth look at bone characteristics and allow for *in vivo* measurements. More recently, digitized fluoroscopy and ultrasound (Fleming, et al., 2004), dual energy X-ray absorptiometry (DEXA) (Hester, et al., 2004) and quantitative computed tomography (QCT) (Korver, et al., 2004) have been used in studying bone biology of the avian species. These are all non-invasive techniques that allow the researcher to study the changes in bone density over time within the same bird.

In poultry studies, the use of QCT might allow for close monitoring of changes in BMD of the individual bone fractions over time, which might not be apparent using destructive measures such as total bone ash, Ca and strength measurements. In humans, QCT cortical and trabecular density and bone mineral content are correlated with bone strength (Alho, et al., 1988; Lochmuller, et al., 2002; Wachter, et al., 2002; Lill, et al., 2003). In addition, although both QCT cortical BMD and area of the human trochanter correlate with bone strength, cortical area has been shown to be a better predictor (Cheng, et al., 1997). In the horse third metacarpal, QCT cross-sectional area was highly correlated with bone mineral content (Waite, et al., 2000). QCT has only recently been introduced for use in poultry bone metabolism research (Korver, et al., 2004; Riczu, et al., 2004; Kim, et al., 2007). Therefore, the objective of this study was to compare QCT BMD and cross-sectional area to traditional methods of bone quality evaluation. We hypothesized that QCT BMD and cross-sectional area would correlate with the traditional methods of bone quality evaluation.

3.2. MATERIALS AND METHODS

3.2.1. Bone Mineral Density and Area

Bone density and area cross-sectional analysis were performed using a Norland Stratec XCT quantitative computed tomography scanner with a 50kV x-ray tube (XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA) as described in by Riczu et al. (2004) and outlined in Chapter 2.

3.2.2. Bone Breaking Strength

Bone breaking strength analysis was performed using a the method described by Riczu et al. (2004). Briefly, an Instron Materials Tester (Model 4411, Instron Corp., Canton, Ma, USA) with Automated Materials Test System software version 8.09, a standard 50 kg load cell, and a modified shear plate (8 cm in length and 1 mm in width) were used. Within each experiment, a uniform distance was set between 2 fixed points supporting the bone, and a crosshead speed of 100 mm/min was held constant throughout each measurement.

3.2.3. Bone Ash and Ca

Bone ash content was determined using the bone ash procedure described by Zhang and Coon (1997). Briefly, bones were oven-dried at 100 C for 16 h, allowed to come to room temperature in a desiccator, and weighed to obtain a dried weight. Dried bones were ashed at 600 C for 24 h in a muffle furnace; the ash was then weighed. Percent ash was calculated as the ratio of ash weight to dried bone weight.

A 200 mg portion of the ashed bone was dissolved in 15 ml of a 1:1 HCl: DDH_2O solution and 10 drops of nitric acid. This solution was then diluted to 1:100ml with DDH_2O and further diluted to 0.5:25ml with 0.5% lanthanum chloride (Fisher Scientific, Nepean, Ontario, Canada) to result in a Ca concentration in the solution between 0 and 5 µg/ml. Standard solutions were prepared from a 1,000 ppm Ca reference solution. Calcium content of the ash solution was determined from standard solutions and samples using a Perkin Elmer Model 5000 atomic absorption spectrophotometer (Perkin-Elmer Model 5000, Norwalk, CT).

3.2.4. Bone Trait Correlation Study

For each of the following 5 experiments, bone measures were collected on chickens (broiler breeders, broilers and laying hens) that were subjects of other individual studies. These experiments were carried out with objectives not related to the work presented in this paper. The goal of the following experiments in this study was to determine relationships of traditional bone quality measures, such as bone weight, breaking strength, ash content and Ca content with QCT BMD and bone cross-sectional area measures within experiments using different types and ages of birds.

3.2.5. Experiment 1

Broiler breeders were housed in individual cages and reared under different day lengths and light intensities. At 29.5 wk of age, broiler breeder hens were observed to be lame and to be laying a significant number of soft-shelled eggs. It was discovered that the hens had not been given a high Ca breeder diet. Upon discovery of the error, feed was immediately top-dressed with oyster shell and at 30 wk, hens were fed a breeder diet containing 3.25% w/w Ca. At 0, 7 and 14 d post-Ca replenishment, 30, 32 and 31 left tibiae per time point, respectively (equally distributed across all prior experimental treatments) were excised and frozen with the flesh on at –20 C for subsequent analysis. After thawing and removal of the soft tissue, tibial mid-shaft total, cortical and trabecular BMD and areas were determined using QCT; tibia ash and Ca were measured as described above.

3.2.6. Experiment 2

Broilers had been fed one of 5 dietary combinations of vitamin D_3 or 25-OH D_3 (HyD, DSM Nutritional Products, Parsippany, NJ) from 0 to 42 d to assess the effect of the dietary treatments on broiler production parameters. At 42 d, femurs were removed from a total of 259 birds and stored at –20 C with the flesh on until further analysis. After thawing, total, cortical and trabecular BMD and areas were measured at the bone mid-shaft using QCT with flesh removed, in addition bone breaking strength, ash and Ca contents were also determined.

3.2.7. Experiment 3

To examine the effect of midnight feeding on egg quality of laying hens, a group of 48 individually-caged laying hens within the same barn were divided into 2 groups of 24 at 63 wk of age. With an additional hour of lighting at midnight, one group had ad libitum access to feed whereas the other group had feed removed when lights were off each night. At 67 wk of age, tibias were collected from all hens and frozen with the flesh on at -20 C until further analysis was completed. After the tibias thawed, they were analyzed for total, cortical and trabecular BMD and areas by QCT analysis at the bone midpoint with the flesh on. The soft tissue was removed, and bone breaking strength, ash and Ca contents were also determined as describe previously.

3.2.8. Experiment 4

Laying hens were fed one of 8 dietary combinations of 4 calcium sources and 2 particle size treatments from 19 to 74 wk of age to assess the effect of calcium source and

particle size on laying hen production and bone quality. Tibias were excised from 16 hens per treatment and frozen at –20 C with the flesh on until further analysis. Following thawing, the flesh was removed and total, cortical and trabecular BMD and areas at the bone mid-shaft with were measured using QCT; bone breaking strength, ash and Ca contents were determined. Bone analysis procedures were performed as outlined previously except that bones were dried at 100 C in a forced air-dried oven for 24 h prior to all bone analysis procedures. As well, bone ash and Ca were determined on fat-extracted (Soxlet method with petroleum ether; (Whitehead, et al., 2004) whole bones.

3.2.9. Experiment 5

Shaver 2000 (white strain; n=24) and Shaver 579 (brown strain; 24) hens were individually housed in cages at 60 wk of age and were assessed for differences in end-of-lay bone quality between the 2 strains (Riczu, et al., 2004). Femurs and humeri from each hen were collected at 65 wk of age and stored at -20 C until further analysis. After thawing and removal of flesh, femurs and humeri were analyzed for total, cortical and trabecular BMD and areas at the bone mid-point by QCT; in addition breaking strength was also measured as described previously.

3.2.10. Statistical Analysis

Linear regression analyses of BMD and bone area measurements using breaking strength, ash, and Ca as the dependent variables were performed using linear regression analysis of SAS (SAS Institute, 1999). The level of significance, unless otherwise stated, was assessed at a probability of $P \leq 0.05$.

3.3. RESULTS AND DISCUSSION

3.3.1. Relationship of QCT BMD and Area with Bone Weight and Breaking Strength

Tibia weight had moderate, but significant, positive linear relationship with total BMD (r^2=0.30; P<0.05; Table 3.1), but was weakly and negatively related to cortical and trabecular BMD in broiler breeders (r^2=0.05 and 0.09, respectively; P<0.05; Table 3.1). However, femur weight had a positive relationship with trabecular BMD of the broiler femur (r^2=0.06; P<0.05; Table 3.1). There were no relationships between bone weight and BMD measurements of laying hen tibias in Experiment 3 (Table 3.1). Bone weight had a positive relationship with trabecular BMD of the laying hen tibia in Experiment 4 (r^2= 0.07, P<0.05) but was negatively related with trabecular BMD in laying hen femur (r^2=0.19; P<0.05; Table 3.1). Similar to broiler breeders, femur weight of laying hens was also negatively related to trabecular BMD (r^2=0.19; P<0.05; Table 3.1).

Bone weight was positively related to total and cortical cross-sectional areas of all broiler breeder (r^2=0.08 and 0.48; respectively; P<0.05; Table 3.1), broiler (r^2=0.12 and r^2= 0.06; respectively; P<0.05; Table 3.1) and laying hen studies (r^2 ranging from 0.12 to 0.50; and 0.09 to 0.42; respectively; Table 3.1). Bone weight was negatively related with trabecular bone cross-sectional area in the broiler breeder tibia (r^2=0.13; P<0.05; Table 3.1) as well as the laying hen tibia in Experiment 4 (r^2=0.04, P<0.05; Table 3.1). In contrast, bone weight was positively related to trabecular bone cross-sectional area of the broiler femur (r^2=0.06; P<.05; Table 3.1). This is most likely due to the difference in bone characteristics between egg-laying and non-egg-laying birds. In egg-laying birds, medullary bone would occupy the trabecular space of both femurs and tibiae (Fleming, et

al., 1998a). As the bird ages, medullary bone stores can increase even as the outer cortical bone becomes thinner due to endocortical mobilization of bone (Fleming, et al., 1998b). However, an increase in trabecular area does not necessarily mean there is an increased amount of bone tissue within the trabecular space. This measure (as it is given by the QCT) is simply a measure of the amount of area in the trabecular space. Therefore increases in trabecular cross-sectional area could be due to endocortical thinning, leading to less cortical bone tissue and a decrease in the overall weight and strength of the bone. In broilers, cortical thinning is not likely to occur because Ca is not required for eggshell formation.

Bone breaking strength was positively related to total BMD in broiler femurs (r^2= 0.49; P<0.05; Table 3.2) and laying hen bones in all studies (r^2 ranging from 0.16 to 0.64; P>0.05; Table 3.2). Cortical BMD were positively related to bone breaking strength in the broiler femur (r^2= 0.14; P<0.05; Table 3.2) and laying hen humerus in Experiment 5 (r^2=0.42; P<0.05; Table 3.2). Trabecular BMD was not positively related to bone breaking strength in any of the studies, however it had a negative relationship with the breaking strength of the laying hen femur in Experiment 6 (r^2=0.11; P<0.05; Table 3.2).

Total cross-sectional area was positively related to breaking strength in the laying hen tibia (r^2=0.14; P<0.05; Table 3.2) and femur (r^2=0.11; P<0.05; Table 3.2). Cortical bone cross-sectional area was positively related to breaking strength in all broiler (r^2= 0.18, P<0.05; Table 3.2) and laying hen studies (r^2 ranging from 0.15 to 0.78; Table 3.2). Unlike cortical bone area, trabecular bone cross-sectional area had a negative relationship with breaking strength in all broiler (r^2= 0.14, P<0.05; Table 3.2) and each of the laying hen studies (r^2 ranging from 0.09 to 0.38; Table 3.2). In Experiment 3, there was a

negative relationship between laying hen trabecular bone area and bone breaking strength of the tibia (r^2=0.36; P<0.05; Table 3.2).

The current study found total BMD to be associated with breaking strength within each of the experiments in which it was performed, with regression coefficients ranging from 0.16 to 0.64 (Table 3.2). Using direct photon absorptiometry in laying hens, Frost and Roland (1991) reported a similar relationship between BMD and breaking strength (r = 0.62; P<0.0001) as those found in most of the current studies. Using DEXA to determine BMD in laying hens, Schreiweis et al. (2005) found a correlation coefficient of 0.65 between BMD and bone breaking strength. Similarly, in humans, Bonnaire *et al.* (2002) reported strength tests of the femoral neck to be correlated with BMD as determined by QCT at the femoral head (r=0.74; P<0.01) and the femoral neck (r=0.51, P <0.01). These studies indicate that although BMD does not explain all of the variation in breaking strength, it does have a significant linear relationship with breaking strength and may therefore be used to assess bone strength.

One of the advantages of QCT is that it measures bone cross-sectional area in additional to BMD. Within the majority of the experiments in the current study, bone area and more specifically cortical area, had a better fitted linear regression with bone breaking strength (r^2 ranging from 0.15 to 0.78) than BMD measurements. This has also been found to be true in studies using QCT analysis on humans. Cheng et al. (1997) reported cortical area of both the femoral neck and femoral trochanteric area to have a stronger relationship with femur strength (r^2=0.66 and 0.83; respectively; P<0.001) and only a weak relationship between femur breaking strength and cortical BMD (r^2=0.07 and 0.28 for the neck and trochanteric area, respectively; P<0.001). A study by Augat et al.

(1996) also reported a higher correlation of radius breaking strength and cortical area ($r = 0.89$, $P<0.0001$) than other BMD and area measurements. This may be due to the fact the bone area measurements provide a greater range of values between subjects, whereas BMD (in healthy subjects) remain fairly close in value, providing a smaller scale from which to draw a relationship. In addition, bone weight (indicative of bird BW) was always positively related to total bone area (Tables 3.1 to 3.5). Riczu et al. (2004), found that although brown egg-laying hens had higher body weights but similar BMD to the white egg-laying hens, bone cross-sectional area and breaking strength were greater for the brown egg strain.

QCT could be advantageous for use in poultry bone quality research. A positive linear relationship was observed between breaking strength and both total density and cortical area in the laying hen midnight feeding study (Experiment 3; Table 3.2) and in the calcium source and particle size laying hen study (Experiment 4; Table 3.2). In addition, the broiler study involving different vitamin D sources and levels (Experiment 2) revealed the usefulness of QCT in broiler bone metabolism studies in which bone weight was related to total and cortical area and breaking strength was related to total and cortical BMD as well as cortical and trabecular areas (Table 3.2). QCT may provide a greater array of information than can be gathered using traditional, destructive bone strength testing methods because the information obtained through QCT allows the researcher to see what is happening in the individual bone sections (ie. cortical and trabecular), not simply a measure of the breaking force of the bone.

3.3.2. Relationship of QCT BMD and Area with Bone Mineral Content

Total BMD was positively related to ash weight of broiler breeder tibias (r^2=0.24; P<0.05; Table 3.3) as well as laying hen tibias (r^2 of 0.20 and 0.09 for Experiments 3 and 4, respectively; P<0.05; Table 3.3). Similarly, % ash (r^2 of 0.22 and 0.06, for Experiments 3 and 4, respectively) and Ca (r^2=0.25 for Experiment 3) were also positively related to total BMD in laying hens (Table 3.4). Cortical BMD was negatively related to ash weight (r^2=0.14; P<0.05) and % ash (r^2=0.07; P<0.05) of broiler breeders (Tables 3.3 and 3.4) as well as % ash of laying hen tibias in Experiment 3 (r^2=0.12; P<0.05; Table 3.4). This relationship was not observed in the laying hen tibias in Experiment 4, possibly due to the fact that the bones were dried prior to BMD analysis which has been shown previously to increase QCT cortical BMD measurement (see Chapter 2). Laying hens have a much greater demand for Ca due to the greater rate of lay than broiler breeders. In addition they have a much smaller bone structure than broiler breeders. Trabecular BMD was negatively related to ash weight in broiler breeder tibias (r^2=0.13; P<0.05; Table 3.3) and % ash of broiler femurs (r^2=0.06; P<0.05; Table 3.4). Trabecular BMD was positively related to ash weight (r^2=0.12; P<0.05), % ash (r^2=0.12; P<0.05) and % bone Ca of laying hen tibias (r^2=0.12; P<0.05) (Tables 3.3 to 3.5). This effect may not have been observed in Experiment 4 laying hen tibias because the bones were dried prior to QCT BMD and cross-sectional area analysis. Previously, it has been shown that drying bones resulted in a significant decrease and/or complete disappearance of trabecular BMD as measured by QCT (see Chapter 2). Therefore, trabecular BMD may have been altered by the drying of the bone prior to the density analysis so that its measurement was not related to ash and Ca content.

Total cross-sectional area was positively related to ash weight in broilers (r^2= 0.17, P<0.05; Table 3.3) and laying hen tibias (r^2=0.13; P<0.05; Table 3.3). However, total cross-sectional area was negatively related to % ash of broiler breeder tibias (r^2=0.07; Table 3.4) and % Ca of broiler breeder (r^2=0.10; Table 3.5) and laying hen tibias (r^2=0.04; P<0.05; Table 3.5). Cortical cross-sectional area was positively related to ash weight of broiler breeder tibias (r^2=0.37; P<0.05; Table 3.3), broiler femurs (r^2=0.11; P<0.05; Table 3.3) and laying hen tibias (r^2 of 0.31 and 0.04 for Experiments 3 and 4, respectively; P<0.05; Table 3.3). In one laying hen study (Experiment 3), % ash and % bone Ca were positively related to cortical cross-sectional area (Tables 3.3 and 3.4), whereas in another laying hen study (Experiment 4), % ash was again positively related to cortical cross-sectional area while cortical cross-sectional area was not related to % bone Ca (Tables 3.3 and 3.4). Trabecular cross-sectional area was negatively related to ash weight, % ash and % Ca of broiler breeder tibias (r^2=0.21, 0.06, and 0.05 respectively; P<0.05; Tables 3.3 to 3.5). However, trabecular cross-sectional area had a positive relationship with ash weight of broiler femurs (r^2= 0.06, P<0.05; Table 3.3). Similar to the broiler breeders, laying hen tibia trabecular cross-sectional areas had a negative relationship with ash weight in both Experiments 3 and 4 (r^2=0.14 and 0.03; P<0.05; Table 3.3), % ash in Experiment 3 (r^2=0.34; P<0.05; Table 3.4) and % bone Ca in Experiment 3 (r^2=0.36; P<0.05; Table 3.5).

Bone ash is a common, relatively simple procedure used to assess bone mineralization in poultry research. However, several different variations of this analysis are reported in the literature, from the method of flesh removal, fat extraction procedures to length of ashing time (Akpe, et al., 1987; Orban, et al., 1993). One drawback to this

procedure is that it can only occur ex vivo and is therefore limited in longitudinal bone quality studies to using different birds across time. It is common to report bone ash as a percent of bone weight. However, researchers have found total ash weight to be more sensitive to bone mineralization and dietary Ca changes than ash represented as a percent of the bone (Cheng and Coon, 1990; Hall, et al., 2003). Similarly, in the current study, ash weight but not % ash was found to have higher positive linear relationships with total BMD in broiler breeders (Tables 3.3 and 3.4). As laying hens age, there is an increase in medullary bone but a decrease in structural bone (Fleming, et al., 1998b; Whitehead, 2004), which could increase the % ash, masking the loss of structural bone if conclusions are drawn based on ash content alone. As medullary bone is higher in Ca content than cortical bone (reviewed by Dacke et al., 1993), the loss of structural bone (cortical bone) at the expense of medullary bone could explain some of the negative relationships of cortical BMD and area and % ash in broiler breeders and laying hens of the current study. It has previously been shown that % tibia and femur ash were not different among hens with and without osteoporosis (Bell and Siller, 1962). In addition, total ash content was also not different between fractured and non-fractured turkey femurs (Crespo, et al., 2002). Therefore the use of bone ash alone may be of limited use in the study of bone quality in poultry.

Ash weight had a weak relationship to total BMD in the current experiments with r^2 of 0.23, 0.22 and 0.09 for the broiler breeder and 2 laying hen studies, respectively (Table 3.3). Quantitative CT results were highly correlated with bone ash weights in horse studies ($r = 0.91$; Waite, et al., 2000). Bone mineral content in human cadaver femurs, as assessed using DEXA, was found to the highly correlated with ash weight

(r=0.87; Lochmuller, et al., 2000). A possible reason for the weaker relationship between ash weight and bone density in the current studies than those found in horses may be attributed to the bone ash analysis of the whole bone in the current studies and only the scanned section in the horse study. Therefore the stronger relationship between density and ash was attributed to the fact that both analyses were measuring the same bone section of the horse. In contrast, using DEXA, Schreiweis et al. (2005), found a strong positive correlation (r=0.77) between BMD and bone ash. As DEXA measures a 2-dimensional average BMD of the entire bone, a stronger relationship to the ash content of the whole bone would be expected.

Bone mineral makes up about 60 to 70% of the bone weight, and consists mostly of Ca and P (Rath, et al., 2000). Cann (1988) reviewed the use of QCT and reported that it was highly correlated with the Ca content of cortical bone in human studies. Although there were no differences in ash weight, non-fractured turkey femurs had significantly higher Ca content than fractured femurs (Crespo, et al., 2002). In the current study, bone Ca content was not strongly related to BMD in any of the experiments. However, a significant, moderate linear relationship of bone Ca and total BMD was found in the laying hen tibia (r^2=0.25; Table 3.5). Total cross-sectional area had a negative, weak linear relationship with bone Ca in broiler breeder (r^2=0.07; Table 3.5), and laying hen tibias (r^2=0.04; Table 3.5) and suggests that within an experiment, larger bones may not necessarily have a greater calcium content, likely due to a similar Ca content spread over a greater bone area. Williams et al. (1998, 2000), demonstrated that fast- and slow-growing broiler strains had similar % bone ash, even though the fast-growing strain had a greater cortical bone cross-sectional area. In addition, the cortical bone of the fast-

growing strain is more porous than slower growing strains (Williams, et al., 2000; Williams, et al., 2004). Similar to the results with broiler breeders and laying hens in the current study, a weak linear relationship between bone Ca and specific mineral density was found in turkeys (r^2=0.19; P=0.024) (Crespo, et al., 2002). It is not known why Ca was not related to BMD in the other experiments as was observed with the broiler breeder and one laying hen study (Experiment 3). The relationship between BMD and Ca content that exists in broiler breeder and laying hen bones would be expected to be different than that of broiler bones due to the effect of high bone remodeling due to demands on bone mineral because of egg production. Broiler breeder and laying hens would have medullary bone present which may affect the relationship of BMD and area. As the hen ages, there is an increase in medullary bone which would increase the Ca content of the bone as it is rich in minerals, however there is also a decrease in structural bone (Fleming, et al., 1998b; Whitehead, 2004). Together the increase in medullary bone would increase bone Ca, however the decrease in structural bone would decrease overall BMD, resulting in the negative relationship of Ca and BMD found in the broiler breeders and laying hens.

QCT BMD and cross-sectional area measurements were found to relate to other common bone quality measurements throughout the various studies with different ages and species of chickens as well as different dietary treatments. Although only low to moderate relationships were observed, it must be remembered that only one specific portion of the bone (a 1 mm slice of the midpoint of the bone) from the QCT measurements was compared to strength, ash and Ca measurements from the whole bone. Although single-point QCT can be used to accurately track changes in bone

characteristics over time, measurements taken within an experiment must be consistent with regards scan location (as determined in Chapter 2).

The comparison of BMD to strength and mineral content measures would also not be expected to have high correlations as they are each measuring different aspects of the bone. However, the fact that relationships did exist between QCT measurements at the midpoint and assessments of the whole bone indicates that QCT would be a valid tool for assessing bone quality in poultry. The results of this study indicate that QCT measurements of bone quality were in agreement with the traditional measures. The positive significant regressions of BMD and area with bone breaking strength, ash and Ca indicate that QCT indicate that this method would classify bones similarly as the traditional bone quality methods. Under research conditions, QCT improves upon these other traditional methods by providing a more in-depth look at bone fractions and makes it possible to examine bone quality *in vivo*, and over time within individual birds. QCT bone quality measures provide a less invasive assessment of bone quality and yields similar conclusions as compared to most traditional bone quality methods.

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Table 3.1. Regression analysis of bone mineral densities and cross-sectional areas as measured by quantitative computed tomography with bone weight.

	Density (mg/cm^3)			Area (mm^2)		
	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Bone Weight						
Experiment 1 – Broiler Breeders[1]						
y-Intercept	311.83	1128.94	242.78	42.82	-6.11	41.94
Slope	34.68	-14.87	-12.12	1.48	4.05	-2.36
SE	5.68	6.55	3.98	0.54	0.44	0.63
r^2	0.30	0.05	0.09	0.08	0.48	0.13
P-value	<0.0001	0.0256	0.0030	0.0079	<0.0001	0.0004
Experiment 2 – Broilers[2]						
y-Intercept	427.29	862.16	49.97	52.67	22.96	26.88
Slope	5.89	-0.18	10.01	4.62	2.12	2.17
SE	4.93	4.38	2.76	0.84	0.53	0.66
r^2	0.01	0.00	0.06	0.12	0.06	0.05
P-value	NS[2]	NS	0.0004	<0.0001	<0.0001	0.0011
Experiment 3 – laying hens[3]						
y-Intercept	537.19	1108.24	155.57	24.51	7.62	14.10
Slope	24.80	-6.94	15.40	2.07	1.96	0.08
SE	20.64	13.23	14.15	0.87	0.9605	1.02
r^2	0.03	0.01	0.03	0.12	0.09	0.00
P-value	NS[6]	NS	NS	0.0217	0.0478	NS
Experiment 4 – laying hens[4]						
y-Intercept	253.79	1188.40	37.15	15.92	-5.64	21.81
Slope	51.06	-19.96	17.13	2.44	3.16	-0.99
SE	9.16	12.62	5.58	0.22	0.34	0.43
r^2	0.20	0.02	0.07	0.50	0.42	0.04
P-value	<0.0001	NS[2]	0.0026	<0.0001	<0.0001	0.0228
Experiment 5 – laying hen femurs[5]						
y-Intercept	513.78	961.20	386.02	19.89	3.53	16.55
Slope	4.33	-3.61	-19.99	2.87	1.92	0.3755
SE	10.31	6.64	6.28	0.59	0.60	0.88
r^2	0.00	0.01	0.19	0.35	0.19	0.00
P-value	NS[2]	NS	0.0027	<0.0001	0.0025	NS
Experiment 5 – laying hen humeri[5]						
y-Intercept	2.37	934.94	N/A[7]	36.58	4.94	30.00
Slope	38.96	11.74	N/A	0.75	1.14	-0.25
SE	10.21	5.93	N/A	0.52	0.23	0.59
r^2	0.27	0.09	N/A	0.05	0.37	0.00
P-value	0.0005	0.0547	N/A	NS	<0.0001	NS

[1]Linear regression analysis of tibia bone mineral density (BMD) and cross-sectional area with bone weight, ash and Ca from excised broiler breeder tibias (29 to 31 wk old) collected at 0, 1 and 2 weeks post-Ca repletion. Thirty bones were excised at each time period, data was combined for regression analysis (n=90).

[2]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 259 femurs collected from 42 d-old broilers. Broilers had been on an experiment in which they received 1 of 5 dietary combinations of vitamin D or 25-OH D_3.

[3]Linear regression analysis of QCT BMD and cross-sectional area measurements at bone midpoint with bone weight, ash weight, % ash, % bone Ca and breaking strength on tibias excised from 48, 63-wk old laying hens fed for either 16 or 24 hours/day with and additional hour of light provided at midnight.

[4]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 128 tibias excised from 74 wk-old laying hens. Laying hens had been on a trial in which 4 different Ca sources and 2 different calcium source particle sizes were tested.

[5]Linear regression analysis of QCT BMD and cross-sectional area with bone weight and breaking strength of 48 femur and humerus bones from 65 wk-old laying hens. Laying hens were from an experiment examining the difference in bone quality of brown and white egg-laying strains.

[6]Not statistically significant (P>0.05).

[7]Not applicable due to lack of bone in the trabecular space.

Table 3.2. Regression analysis of bone mineral densities and cross-sectional areas as measured by quantitative computed tomography with bone breaking strength.

	Density (mg/cm³)			Area (mm²)		
	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Bone Breaking Strength						
Experiment 2 – Broilers[1]						
y-Intercept	264.65	772.19	73.07	73.76	19.20	49.72
Slope	6.18	2.94	0.64	-0.05	0.41	-0.45
SE	0.42	0.48	0.33	0.11	0.06	0.07
r^2	0.49	0.14	0.02	0.00	0.18	0.14
P-value	<0.0001	<0.0001	0.0582	NS[5]	<0.0001	<0.0001
Experiment 3 – laying hens[2]						
y-Intercept	409.07	1089.16	202.21	28.61	3.66	23.08
Slope	10.27	-0.48	0.88	0.21	0.54	-0.38
SE	1.18	1.25	1.34	0.08	0.04	0.08
r^2	0.64	0.00	0.01	0.14	0.78	0.36
P-value	<0.0001	NS	NS	0.0116	<0.0001	<0.0001
Experiment 4 – laying hens[3]						
y-Intercept	508.64	1035.47	148.12	34.31	13.25	18.67
Slope	8.59	-0.22	1.38	0.04	0.35	-0.27
SE	1.77	2.40	1.08	0.06	0.08	0.08
r^2	0.16	0.00	0.01	0.00	0.15	0.09
P-value	<0.0001	NS	NS	NS	<0.0001	0.0007
Experiment 5 – laying hen femurs[4]						
y-Intercept	430.12	942.45	266.11	39.08	9.55	26.31
Slope	4.79	-0.50	-2.13	0.23	0.42	-0.26
SE	1.24	0.92	0.91	0.10	0.07	0.12
r^2	0.25	0.01	0.11	0.11	0.47	0.10
P-value	0.0004	NS	0.0241	0.0220	<0.0001	0.0342
Experiment 5 – laying hen humeri[4]						
y-Intercept	6.50	907.60	N/A[6]	40.67	4.99	33.89
Slope	14.19	6.17	N/A	-0.05	0.39	-0.39
SE	1.88	1.17	N/A	0.13	0.04	0.13
r^2	0.59	0.42	N/A	0.00	0.71	0.18
P-value	<0.0001	<0.0001	N/A	NS	<0.0001	0.0056

[1]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 259 femurs collected from 42 d-old broilers. Broilers had been on an experiment in which they received 1 of 5 dietary combinations of vitamin D or 25-OH D_3.

[2]Linear regression analysis of QCT BMD and cross-sectional area measurements at bone midpoint with bone weight, ash weight, % ash, % bone Ca and breaking strength on tibias excised from 48, 63-wk old laying hens fed for either 16 or 24 hours/day with and additional hour of light provided at midnight.

[3]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 128 tibias excised from 74 wk laying hens. Laying hens had been on a trial in which 4 different Ca sources and 2 different calcium source particle sizes were tested.

[4]Linear regression analysis of QCT BMD and cross-sectional area with bone weight and breaking strength of 48 femur and humerus bones from 65 wk laying hens. Laying hens were from an experiment examining the difference in bone quality of brown and white egg-laying strains.

[5]Not statistically significant (P>0.05).

[6]Not applicable due to lack of bone in the trabecular space.

Table 3.3. Regression analysis of bone mineral densities and cross-sectional areas as measured by quantitative computed tomography with bone ash weight.

	Density (mg/cm³)			Area (mm²)		
	Total	Cortical	Trabecular	Total	Cortical	Trabecular
Ash Weight						
Experiment 1 – Broiler Breeders[1]						
y-Intercept	398.35	1168.62	235.79	54.23	4.56	42.27
Slope	42.42	-32.44	-19.13	0.38	4.86	-4.03
SE	8.08	8.55	5.34	0.78	0.67	0.83
r^2	0.24	0.14	0.13	0.00	0.37	0.21
P-value	<0.0001	0.0003	0.0006	NS[5]	<0.0001	<0.0001
Experiment 2 – Broilers[2]						
y-Intercept	439.41	850.01	85.84	49.37	20.82	25.36
Slope	6.52	5.76	3.31	11.60	5.62	5.43
SE	10.39	9.19	5.96	1.72	1.09	1.36
r^2	0.00	0.00	0.00	0.17	0.11	0.06
P-value	NS	NS	NS	<0.0001	<0.0001	<0.0001
Experiment 3 – laying hens[3]						
y-Intercept	439.24	1160.88	111.74	26.20	3.81	22.84
Slope	75.92	-30.57	40.86	2.69	4.54	-3.09
SE	23.53	15.94	16.82	1.08	1.05	1.19
r^2	0.20	0.08	0.12	0.13	0.31	0.14
P-value	0.0024	NS	0.0195	0.0168	<0.0001	0.0127
Experiment 4 – laying hens[4]						
y-Intercept	454.56	1019.17	124.76	34.48	13.22	19.15
Slope	61.79	3.88	14.47	0.18	1.85	-1.58
SE	17.49	22.80	10.27	0.56	0.78	0.77
r^2	0.09	0.00	0.02	0.00	0.04	0.03
P-value	0.0006	NS	NS	NS	0.0186	0.0427

[1]Linear regression analysis of tibia bone mineral density (BMD) and cross-sectional area with bone weight, ash and Ca from excised broiler breeder tibias (29 to 31 wk old) collected at 0, 1 and 2 weeks post-Ca repletion. Thirty bones were excised at each time period, data was combined for regression analysis (n=90).

[2]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 259 femurs collected from 42 d-old broilers. Broilers had been on an experiment in which they received 1 of 5 dietary combinations of vitamin D or 25-OH D_3.

[3]Linear regression analysis of QCT BMD and cross-sectional area measurements at bone midpoint with bone weight, ash weight, % ash, % bone Ca and breaking strength on tibias excised from 48, 63-wk old laying hens fed for either 16 or 24 hours/day with and additional hour of light provided at midnight.

[4]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 128 tibias excised from 74 wk laying hens. Laying hens had been on a trial in which 4 different Ca sources and 2 different calcium source particle sizes were tested.

[5]Not statistically significant (P>0.05).

Table 3.4. Regression analysis of bone mineral densities and cross-sectional areas as measured by quantitative computed tomography with ash weight as a percent of dried bone weight.

	Density (mg/cm³)			Area (mm²)		
	Total	Cortical	Trabecular	Total	Cortical	Trabecular
% Ash						
Experiment 1 – Broiler Breeders[1]						
y-Intercept	580.13	1178.48	201.33	71.98	27.17	37.61
Slope	0.79	-3.11	-1.16	-0.2644	0.06	-0.29
SE	1.26	1.21	0.77	0.10	0.12	0.12
r^2	0.00	0.07	0.02	0.07	0.00	0.06
P-value	NS[5]	0.0122	NS[5]	0.0115	NS	0.0222
Experiment 2 – Broilers[2]						
y-Intercept	472.93	851.42	138.38	66.61	29.06	33.24
Slope	-0.44	0.21	-0.98	0.12	0.06	0.06
SE	0.47	0.42	0.26	0.08	0.05	0.06
r^2	0.00	0.00	0.06	0.01	0.01	0.00
P-value	NS	NS	0.0003	NS	NS	NS
Experiment 3 – laying hens[3]						
y-Intercept	318.34	1228.37	55.28	28.89	-0.55	34.12
Slope	5.23	-2.41	2.68	0.07	0.27	-0.32
SE	1.51	1.02	1.09	0.07	0.07	0.07
r^2	0.22	0.12	0.12	0.02	0.25	0.34
P-value	0.0012	0.0224	0.0185	NS	0.0005	<0.0001
Experiment 4 – laying hens[4]						
y-Intercept	82.79	883.67	82.47	24.82	-6.48	28.18
Slope	10.10	2.62	1.58	0.18	0.45	-0.25
SE	3.73	4.76	2.16	0.12	0.16	0.16
r^2	0.06	0.00	0.00	0.02	0.06	0.02
P-value	0.0077	NS	NS	NS	0.0055	NS

[1]Linear regression analysis of tibia bone mineral density (BMD) and cross-sectional area with bone weight, ash and Ca from excised broiler breeder tibias (29 to 31 wk old) collected at 0, 1 and 2 weeks post-Ca repletion. Thirty bones were excised at each time period, data was combined for regression analysis (n=90).

[2]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 259 femurs collected from 42 d-old broilers. Broilers had been on an experiment in which they received 1 of 5 dietary combinations of vitamin D or 25-OH D_3.

[3]Linear regression analysis of QCT BMD and cross-sectional area measurements at bone midpoint with bone weight, ash weight, % ash, % bone Ca and breaking strength on tibias excised from 48, 63-wk old laying hens fed for either 16 or 24 hours/day with and additional hour of light provided at midnight.

[4]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 128 tibias excised from 74 wk laying hens. Laying hens had been on a trial in which 4 different Ca sources and 2 different calcium source particle sizes were tested.

[5]Not statistically significant (P>0.05).

Table 3.5. Regression analysis of bone mineral densities and cross-sectional areas as measured by quantitative computed tomography with calcium weight as a percent of bone ash.

	Density (mg/cm³)			Area (mm²)		
	Total	Cortical	Trabecular	Total	Cortical	Trabecular
% Ca						
Experiment 1 – Broiler Breeders[1]						
y-Intercept	574.70	1038.42	158.20	67.90	32.78	30.70
Slope	2.24	-1.91	-1.09	-0.50	-0.09	-0.44
SE	1.98	1.97	1.22	0.16	0.18	0.20
r^2	0.01	0.01	0.01	0.10	0.00	0.05
P-value	NS[5]	NS	NS	0.0023	NS	0.0278
Experiment 2 – Broilers[2]						
y-Intercept	445.47	870.07	101.20	69.38	29.32	36.46
Slope	0.47	-0.60	-0.61	0.20	0.18	-0.03
SE	1.18	1.04	0.68	0.21	0.13	0.16
r^2	0.00	0.00	0.00	0.00	0.01	0.00
P-value	NS	NS	NS	NS	NS	NS
Experiment 3 – laying hens[3]						
y-Intercept	337.79	1213.69	78.13	27.79	0.47	32.34
Slope	15.20	-6.71	7.14	0.28	0.77	-0.8856
SE	4.03	2.76	2.99	0.20	0.19	0.18
r^2	0.25	0.12	0.12	0.04	0.28	0.36
P-value	0.0005	0.0194	0.0215	NS	0.0002	<0.0001
Experiment 4 – laying hens[4]						
y-Intercept	633.61	1002.86	189.11	36.80	18.82	16.14
Slope	0.76	1.05	-0.64	-0.06	0.0142	-0.08
SE	0.99	1.23	0.56	0.03	0.0429	0.04
r^2	0.00	0.00	0.01	0.04	0.00	0.03
P-value	NS	NS	NS	0.0347	NS	NS

[1]Linear regression analysis of tibia bone mineral density (BMD) and cross-sectional area with bone weight, ash and Ca from excised broiler breeder tibias (29 to 31 wk old) collected at 0, 1 and 2 weeks post-Ca repletion. Thirty bones were excised at each time period, data was combined for regression analysis (n=90).

[2]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 259 femurs collected from 42 d-old broilers. Broilers had been on an experiment in which they received 1 of 5 dietary combinations of vitamin D or 25-OH D_3.

[3]Linear regression analysis of QCT BMD and cross-sectional area measurements at bone midpoint with bone weight, ash weight, % ash, % bone Ca and breaking strength on tibias excised from 48, 63-wk old laying hens fed for either 16 or 24 hours/day with and additional hour of light provided at midnight.

[4]Linear regression analysis of QCT BMD and cross-sectional area with bone weight, ash weight, % ash, % bone Ca and breaking strength on 128 tibias excised from 74 wk laying hens. Laying hens had been on a trial in which 4 different Ca sources and 2 different calcium source particle sizes were tested.

[5]Not statistically significant (P>0.05)

CHAPTER 4: The Effect of Dietary Vitamin D Source on Plasma 25-OH D_3, Broiler Production, Carcass Composition, and Bone Quality

4.1. INTRODUCTION

Vitamin D is required for growth, health and bone development in the chick. In Canada, broiler production typically takes place in light-tight barn facilities. This impairs the UV-dependent synthesis of vitamin D that takes place in animals exposed to the sun (Norman and Hurwitz, 1993). Therefore a dietary source of vitamin D_3 is necessary for broiler production.

Vitamin D_3 is hydroxylated in the liver by 25-hydroxylase to form 25-hydroxychocalciferol (25-OH D_3) (Soares, et al., 1995). This metabolite is then hydroxylated to 1,25$(OH)_2D_3$ in the kidney by 25-hydroxy-D_3-1α-hydroxylase (Norman and Hurwitz, 1993). 1,25$(OH)_2D_3$ is a steroid hormone (Norman, 1968), and it is through this final metabolite that vitamin D exerts its actions on calcium metabolism and cellular differentiation (Norman and Hurwitz, 1993).

The most well-studied function of vitamin D_3 within the chick is its involvement in calcium metabolism and therefore its involvement in bone development and maintenance. The active vitamin D metabolite, 1,25$(OH)_2D_3$, is involved in the deposition of skeletal minerals, of which Ca comprises the greatest amount, as well as Ca resorption from bone tissues when plasma Ca levels are low (DeLuca, 2004). 1,25$(OH)_2D_3$ and 24, 25$(OH)_2D_3$) have also been shown to be required for the proper development of the growth plate (cartilage) cells (Ornoy, et al., 1978). Vitamin D is therefore crucial in Ca homeostasis so that Ca can be available for bone formation and other critical functions.

The rate of bone development does not match the fast growth rates of modern commercial broilers (Rath, et al., 2000). Maximum bone density and breaking strength are not reached until 35 wk of age in broilers (Rath, et al., 2000), long after the birds are typically marketed. Several developmental problems can arise from poorly-formed bones, each of which can impair the welfare of the broiler and lead to mortality, culls or carcass downgrades. Growth rate affects the way in which the bone organic matrix is formed. With rapid growth rate in broilers, the ability to form a tight, compact bone matrix may be impaired, leading to larger pores within the bone matrix, which weaken the bone structure (Thorp and Waddington, 1997; Williams, et al., 2000). In the past, fast growth rate of commercial broilers have also been implicated in the increased incidence of bone developmental issues (Lilburn, 1994; Rath, et al., 2000). Therefore, bone quality of broilers is of both welfare and economic concern affecting many aspects of the poultry industry, from the bird to the processors.

The most abundant circulating form of vitamin D is 25-OH D_3, plasma levels of which give a good indication of the vitamin D status of the chick (Haussler and Rasmussen, 1972). When either vitamin D_3 or 25-OH D_3 amounts are increased in the diet of the bird, the circulating level of 25-OH D_3 also increases (Yarger, et al., 1995b). However, feeding increasing doses of dietary 25-OH D_3 resulted in a more rapid rate of increase of plasma 25-OH D_3 than did vitamin D_3 diet at similar vitamin D_3 activity levels (Yarger, et al., 1995b).

The natural hepatic production of 25-OH D_3 can become impaired, either due to stress such as infection, mycotoxin feed toxicity (Waldenstedt, 2006) or perhaps due to immaturity of enzyme development in the young chick (Ward, 2004). Therefore the

opportunity exists to improve the vitamin D status of the chick by feeding dietary 25-OH D_3. Currently, 25-OH D_3 is commercially available for use in poultry diets under the trade name HyD®. This product is safe for use in poultry and shows no signs of toxicity at up to 10 times the recommended feeding concentration of 69 µg/kg (Yarger, et al., 1995a). Providing the chick with a dietary source of 25-OH D_3 may allow it to be readily available for the conversion to $1,25(OH)_2D_3$ and therefore offer the potential to enhance the functions that vitamin D metabolites serve within the body. Previous studies have shown dietary 25-OH D_3 to increase BW (Yarger, et al., 1995b; Mitchell, et al., 1997; Aburto, et al., 1998), improve feed conversion efficiency and to increase breast muscle yield in broilers (Yarger, et al., 1995b) in comparison with vitamin D_3. Therefore, the objectives of the current research were to investigate the effects of dietary 25-OH D_3 (and age at receiving dietary 25-OH D_3) on broiler production traits, plasma 25-OH D_3, bone formation and quality and carcass composition at 6 wk of age. It was hypothesized that dietary 25-OH D_3 would enhance broiler production traits and bone quality as compared to dietary vitamin D_3.

4.2. MATERIALS AND METHODS

4.2.1. *Experimental Diets*

Diets were formulated to meet or exceed current NRC recommendations (National Research Council, 1994) and were based on the primary breeder recommendations for Ross 308 mixed-sex broilers (Table 4-1). A 3 phase feeding program with starter (0 to 10 d), grower (11 to 28 d) and finisher (29 to 41 d) phases was used (Table 4-1). For each of the 3 phases, a basal diet devoid of supplemental vitamin D activity was mixed and supplemented with either vitamin D_3 or 25-OH D_3. The Control

treatment received a diet containing 2,760 IU of vitamin D_3 per kg of feed from 0 to 41 d. The 25D treatment received a diet containing 69 µg of 25-OH D_3 per kg of feed, which is the equivalent of 2,760 IU of vitamin D_3 activity, from 0 to 41 d. The 25D Early group received the 25D diet from 0 to 28 d and the Control diet from 29 to 41 d. The 25D Late group received the Control diet from 0-28 d and the 25-OH D_3 diet from 29 to 41 d. Each dietary treatment was replicated 8 times, being fed to 4 pens of males and 4 pens of females.

4.2.2. ***Experimental Conditions***

This experimental protocol was approved by the University of Alberta Faculty Animal Policy and Welfare Committee under the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993). Day old, Ross 308 broiler chicks were obtained from a commercial hatchery, randomly allocated to 32 floor pens at a rate of 110 chicks per pen (15.5 birds/m^2) and grown sex-separately for 41 d. All pens were bedded with clean straw at the start of the experiment. Birds were raised in a light-tight barn with incandescent lighting provided 23 $h \cdot day^{-1}$ and had *ad libitum* access to feed (mash form) and water (nipple drinkers). At days 0, 10, 28 and 41, BW on a pen basis was obtained and feed consumption measured for the starter, grower and finisher phases, respectively. Feed conversion ratio (g feed/g gain) was calculated.

At 41 d of age, 80 male birds from each treatment were selected at random for processing; feed and water were withdrawn for 12 h prior to slaughter. Each bird was uniquely identified with a wing band and individual live BW were measured immediately prior to processing. Birds were processed in a federally-inspected processing plant at the University of Alberta Poultry Research Center and carcass traits assessed. Individual

weight of total carcass, *Pectoralis major (P. major)*, *Pectoralis minor (P. minor)*, wings, thighs and drums were obtained. Percent of yield for each carcass component was calculated as the percent of eviscerated carcass.

4.2.3. *Plasma 25-OH D_3*

Blood samples were collected by brachial venipuncture, except at d 0, when blood was collected through decapitation. Blood samples were obtained at days 0 (n = 10 male and 10 female), 10 (n = 16 birds per treatment per sex), 28 (n = 16 birds per treatment per sex) and 41 (n= 8 birds per treatment per sex). The blood samples were centrifuged at 4,000 x g for 15 min, the plasma was removed and stored at -20 C until further analysis by HPLC. 25-OH D_3 was extracted from plasma as described by Aksnes (1992). Briefly, 0.5 ml of methanol-isopropanol (9:1, v/v) was added to 0.5 ml of thawed plasma, after which 1.5 ml of hexanes was added and shaken for 2 minutes. The samples were then centrifuged at 3,500 x g for 10 min and the top hexane layer removed and evaporated to dryness under N_2. Samples were then reconstituted in 110 µl of methanol, centrifuged at 3,500 x g for 7 minutes and supernatants transferred to HPLC vials. Each sample (50 µl) was injected into a 5 µm C-18 column (15 cm x 4.6 mm; Supelcosil™ LC-18) with a guard column. A gradient at a flow rate of 2.3 $ml \cdot min^{-1}$ was used. A standard curve was obtained using dilutions of a 25-OH D_3 standard.

4.2.4. *Femur Cross-sectional Area and Bone Mineral Density*

Femur samples were collected at days 0, 10, 28 and 41 from the same birds from which the blood samples were obtained and stored at -20 C until further analysis. Femur bone mineral density (BMD) and cross-sectional area analyses were performed on excised right femurs of male and female broilers. In addition, the right femur of the

processed male broilers at 42 d of age were collected from each bird for analysis of bone mineral density (BMD; N = 60 per treatment), bone length, and bone weight (N = 60 per treatment). BMD was assessed using Quantitative Computed Tomography (QCT) with a Stratec Norland XCT scanner having a 50 kV x-ray tube as per the method described by Riczu et al. (2004).

4.2.5. *Femur Breaking Strength*

Breaking strength analysis (N=20 per treatment) was performed using a modified version of the method described in Fleming et al. (1998). Briefly, an Instron Materials Tester with Automated Materials Test System software version 8.09, a standard 2 kN load cell, and a modified sheer plate (8 cm in length and 1 mm in width) were used. Within each age group, a uniform distance was set between 2 fixed points supporting the bone; a crosshead speed of 100 mm/min was held constant throughout each measurement.

4.2.6. *Statistical Analysis*

The experimental unit was the pen for production data. The individual bird was the experimental unit for plasma 25-OH D_3, BMD and area, and processing data. From 0 to 28 d, all data were analyzed as a 2 X 2 factorial with 2 dietary treatments and 2 sexes (except the plasma 25-OH D_3 data). From 29 to 41 d, data were analyzed as a 4 x 2 factorial with 4 dietary treatments and 2 sexes. To make graphical representation clearer, plasma 25-OH D_3 data were analyzed as a 4 x 2 factorial with 4 dietary treatments and 2 sexes from 0 to 42 d. Processing data were analyzed as a 1-way analysis of variance with 4 dietary treatments. In addition, 42 d male bone quality data were analyzed using BW as a covariate. All data were analyzed using the Mixed model analysis of SAS and

significance was assessed at a probability of $P \leq 0.05$ (SAS Institute, 1999). The repeated measures analysis of SAS was performed on the plasma 25-OH D_3 to determine differences at different bird ages (SAS Institute, 1999).

4.3. RESULTS AND DISCUSSION

4.3.1 *Production Performance from D0 to 41*

Broiler BW was similar between dietary treatment groups at 0 and 10 d (Table 4-2). However, at 28 d the birds fed the dietary 25D had greater BW than those fed the Control diet ($P<0.01$; Table 4-2). Birds fed 25D throughout the entire trial had greater BW at 41 d than birds in the Control or 25D Late groups; birds in the 25D. Early group were intermediate to and not different from any of the other treatments (Table 4-2). These results are in agreement with those of Yarger et al. (1995b), who reported that dietary 25-OH D_3, increased final BW when comparing dietary 25-OH D_3 and vitamin D_3 across a range of levels of vitamin activity, including the levels used in the current study. This effect has also be reported by other studies in which broiler BW were greater in broilers either supplemented solely with 25-OH D_3 or in addition to dietary vitamin D_3 when compared with vitamin D_3 alone (Mitchell, et al., 1997; Aburto, et al., 1998). In contrast, Bar et al. (2003), found only broiler BW at 7 and 22 d of age was increased by dietary 25-OH D_3, and only when the diet was slightly restricted in P. This effect was not observed when diets sufficient in P were fed (Bar, et al., 2003), as the level of vitamin D required by the bird may be altered by the levels of other nutrients such as Ca and P in the diet (Whitehead, et al., 2004).

Although there were no treatment effects on BW gain, feed consumption or feed conversion ratio for the starter phase, during the grower phase, the 25D-fed broilers gained more weight per day in addition to an increase in feed consumption, which resulted in a nearly significant decrease in feed conversion (P=0.0619; Table 4-2). During the finisher phase, there were no differences in BW gain due to diet; however broilers from the 25D treatment continued to consume the most feed (Table 4-2). Interestingly, the birds on the 25D Early treatment, that were switched to the vitamin D_3 diet for this period, were now eating less than those on 25D treatment that was still eating the 25-OH D_3 diet. Overall FCE was not different among the treatment groups (Table 4-2). Through a series of 10 experiments comparing dietary 25-OH D_3 and vitamin D_3 across a range of vitamin D activity, Yarger et al. (1995b), found that broilers fed dietary 25-OH D_3 had better feed conversion in the majority of the studies.

Between 10 and 14 d of age, approximately 3.3% of chicks placed were diagnosed with rickets by a veterinarian. While treatments of liquid D_3 and 25-OH D_3 were being arranged (to maintain experimental dietary treatments), clinical signs subsided by 15-16 d and therefore no treatment was administered. This outbreak affected all treatment groups equally, in terms of number of dead and culled chicks, with an average of 4.88% cull rate across all treatments, with a range of 4.46 to 5.49%. The authors do not believe that this outbreak of rickets was due to a nutrient deficiency in the diet, as feed analysis showed appropriate levels of Ca and total phosphorus (Table 4-1). The lack of a treatment effect suggests that it was not associated with a lack of dietary vitamin D activity in either treatment. It is worth noting, that at the same time we obtained chicks, other commercial broilers farms in the area were also reporting chicks with the same

symptoms. In those situations, the birds were treated with liquid vitamin D_3; interestingly, the time course of recovery was similar to our non-treated research flock.

The male birds had a greater hatch BW than the female birds (Table 4-2). At 10 d of age the females had the greatest BW, although by 28 d and continuing until the final BW at 41 d, the males again had greater BW (Table 4-2). The greater BW of the females at 10 d of age can be explained by the greater rate of gain of these birds during the starter period (Table 4-2). Similarly, the males had the greatest gain for the grower and finisher periods which resulted in the greater BW of the males at these ages (Table 4-2). Similarily, previous research has reported greater BW of male broilers at 6, 7, 10 and 12 weeks of age when compared to females with the same strains (Edwards, et al., 1973; Leeson and Summers, 1980; Havenstein, et al., 1994).

Feed consumption was not different between the male and female chicks during the starter phase (0 to 10 d; P=0.89; Table 4-2). However for the remainder of the trial the male broilers consumed significantly more feed than the females (P<0.0001; Table 4-2). Feed conversion was lowest for the female birds during the starter period due to the greater rate of gain and similar feed consumption (Table 4-2). However, although the males tended to eat more feed during the grower and finisher, due to the greater rate of BW gain, the males also had a lower feed conversion overall and for the grower and finisher periods (Table 4-2). Past research has also shown male broilers to be more efficient than females, having lower feed conversions at 6, 7, 10 and 12 wk of age for birds of the same strain (Edwards, et al., 1973; Leeson and Summers, 1980; Havenstein, et al., 1994). There were no interactions of diet and sex in the current study.

4.3.2. *Plasma 25-OH D_3*

There was no difference in plasma 25-OH D_3 at hatch among the 10 female and 10 male birds randomly chosen before placement in floor pens. At 10 d of age the chicks fed the dietary 25D had a greater than 2-fold higher amount of circulating 25-OH D_3 than the chicks fed vitamin D_3 (Figure 4-1; $P<0.0001$). From 0 to 10 d plasma 25-OH D_3 decreased among birds fed vitamin D_3, from 10.64 to 5.32 ng/ml ($P<0.0001$; Figure 4-1). However, chicks fed 25D from 0 to 10 d had similar plasma 25-OH D_3 levels at both time points (10.64 at 0 d and 13.88 ng/ml at 10 d; Figure 4-1). The blood chemistry at 10 d of age demonstrated that 25-OH D_3 was present at detectable levels, and was at a greater level in the birds that were on the 25D treatments than the vitamin D_3 treatments.

By 28 d, birds in both treatment groups had increased plasma 25-OH D_3 from the 10 d levels, ranging from 1.8 to 3 times the 10 d sample levels ($P<0.0001$; Figure 4-1). However, the birds that received dietary 25D still had a greater plasma 25-OH D_3 level than birds fed the Control ration ($P<0.0001$; Figure 4-1). These results seem to indicate impairment either in the conversion of dietary vitamin D_3 to 25-OH D_3 or absorption of vitamin D from the gut between 0 and 10 d of age. In support of this, previous research by Stevens et al (1984), found that kidney 1-hydroxylase activity peaked at 8-12 d in the progeny of poults from turkeys consuming 2,700 IU of vitamin D_3. Kidney 1-hydroxylase activity is greatest when vitamin D_3 is deficient (Stevens, et al., 1984). Taken together, these results show there was lower production of vitamin D_3 metabolites by the vitamin D_3-fed chicks, potentially due to inadequacies in the liver conversion of vitamin D_3 to 25-OH D_3 until about 10 d of age.

At 28 d, the birds in the 25D Early treatment were switched to the Control diet and the 25D Late birds were switch to the 25-OH D_3 diet. This diet change resulted in a

decrease in the plasma 25-OH D_3 levels of the birds in the 25D Early treatment, and an increase in plasma 25-OH D_3 levels in birds on the 25D Late treatment (Figure 4-1). Bar et al., (1980) reported that 25-OH D_3 is more readily absorbed than vitamin D_3 from the intestinal tract of the chick. This may in part be due to the greater binding affinity of 25-OH D_3 with the vitamin D binding protein than vitamin D_3 (Soares, et al., 1995). In addition, it has also been suggested that the absorption of 25-OH D_3 is less fat dependent than vitamin D_3 (Ward, 2004). The greater 25-OH D_3 plasma levels in the birds fed dietary 25-OH D_3 than vitamin D_3, in the current study, may indicate either a difference in the absorption of these nutrients from the gut or quite possibly an impairment in the hydroxylation of vitamin D to 25-OH D_3 in the liver. The results of the current study are in agreement with previous research by Yarger et al. (1995b) who reported that plasma levels of 25-OH D_3 increased at a greater rate when birds were fed increasing levels of 25-OH D_3 than dietary vitamin D_3. Mitchell et al. (1997) also reported increases in plasma 25-OH D_3 when dietary 25-OH D_3 was provided to broilers. These results indicate that dietary 25-OH D_3 is effective at increasing circulating plasma levels of 25-OH D_3.

There was no effect of sex on circulating levels of 25-OH D_3 at 0, 10, 28 or 41 d of age, nor was there an interaction of sex and diet (data not shown). Previous research on plasma 25-OH D_3 levels in chickens have not investigated the effect of sex, however from the current study we can conclude that plasma levels of 25-OH D_3 are not influenced by sex when fed the same level of either vitamin D or 25-OH D_3.

4.3.3. ***Femur Bone Mineral Density***

At 10 d of age, total femur BMD was greatest for the birds in the 25D treatment group, as a result of a greater cortical density (P= 0.0065; Table 4-3). The 25D treatment group also had a marginally lower trabecular density (P=0.0570; Table 4-3). These birds not only had a greater cortical BMD but also a greater femur cortical cross-sectional area (Table 4-3), indicating an increase in structural bone deposition. At 10 d of age there were no treatment effects on total or trabecular cross-sectional area, femur weight or length (Table 4-3). Both total and cortical BMD were greater at 28 d of age for femurs from the broilers receiving dietary 25D than those of the birds fed vitamin D_3 (Table 4-3). Cortical bone provides the majority of the bone strength (Whitehead and Fleming, 2000), therefore with a greater cortical BMD these birds would likely have increased bone strength. Unlike the treatment effects at d 10, the 25D treatment resulted in a lower trabecular bone cross-sectional area; total and cortical cross-sectional areas were not affected at 28 d. Similar to 10 d of age, there were no dietary treatment effects on femur weight or length at 28 d (Table 4-3). By 41 d of age there were no dietary treatment effects on any BMD, cross-sectional area measurements or femur weight, however bone length was greatest for the Control group versus the 25D Early treatment, but not different than the 25D and the 25D Late treatments (Table 4-3). The biological significance of a longer femur or why it was greatest for the Control at 41 d of age is not known at this time.

Among the male broilers selected for processing at 42 d of age, dietary supplementation of 25-OH D_3, either for the entire production period, or for the first 28 d of life, had a positive effect on total BMD (P=0.0032), cortical cross-sectional area (P=0.0077), as well as bone breaking strength (P=0.0006; Table 4-4). The increased

cortical cross-sectional area, with a similar cort al density of the femurs from the male broilers would be expected to increase the breaking strength as breaking strength and cortical area are positively correlated (r = 0.85) (Jepsen, et al., 2003). However, when BW was used as a covariate, as the birds from the 25D treatment group had a greater BW, cortical cross-sectional area and bone breaking strength were no longer different between the birds from the control and 25D treatments (Table 4-4). However, total and cortical BMD was greatest for the birds from the 25D and 25D Early treatment groups when BW was used as a covariate (Table 4-4). The discrepancy between the results from the femur density analysis of the male birds selected for processing and the birds sampled at 41 d could simply be due to the number of samples. There were only 16 birds per treatment sampled at 41 d for femur bone analysis (n = 8 male and 8 female) (Table 4-3), whereas there was a much greater number of femurs collected from the male broilers that were processed at 42 d (n = 48 to 51 per treatment; Table 4-4).

The results of the current study indicate that chickens from the 25D treatment had greater femur cortical area and BMD and improved bone strength (Tables 4-3 and 4-4) than birds receiving vitamin D_3. Studies comparing the effect of vitamin D_3 and 25-OH D_3 on bone characteristics have been conflicting and could be a result of the different vitamin D and 25-OH D_3 concentrations used as well as the levels of other nutrients such as Ca and P. It has been shown that biological responses to dietary vitamin D_3 are dependent on Ca and P levels, whereby limiting levels of either mineral increases the vitamin D requirement of the chick (Whitehead, et al., 2004). Although no reported studies have investigated the effect of vitamin D source on bone characteristics as birds age, several have reported that vitamin D and its metabolites positively affect various

bone characteristics of the broiler at specific ages (McNaughton, et al., 1977; Applegate, et al., 2003; Fritts and Waldroup, 2003). Bar et al. (2003) found no difference in the amount of bone ash from broilers fed either dietary vitamin D_3 or 25-OH D_3 in experiments designed to examine the use of 25-OH D_3 either as a complete replacement of dietary vitamin D_3, in addition to dietary vitamin D_3 (at both adequate and deficient levels). The single experiment by Bar et al. (2003) which showed a greater tibia ash due to 25-OH D_3 was when Ca and P were slightly restricted. Similarly, Ledwaba and Roberson (2003), found 25-OH D_3 to be most effective at reducing the severity of TD when dietary Ca was low. This suggests that 25-OH D_3 may be more potent than vitamin D at protecting the bird from deficiencies of Ca and P. Unlike Bar et al. (2003) or Ledwaba and Roberson (2003), the results of the current study showed that 25-OH D_3 improved BMD and cortical cross-sectional area even at sufficient levels of dietary Ca and P. In the current study, bone quality was assessed using BMD and cross-sectional area as well as bone strength whereas those reported previously measured bone ash or TD scores (Bar, et al., 2003; Ledwaba and Roberson, 2003). The differences in the types of measurements taken may have affected the conclusions drawn. Total bone ash and TD incidence may not be as sensitive to change as QCT, which measures both the density and cross-sectional distribution of bone mineral.

At hatch, there was no effect of sex on femur BMD; however female birds had a greater femur total cross-sectional area and length than the males (Table 4-3). The female chicks had a greater cortical cross-sectional area at 10 d of age ($P<0.05$; Table 4-3) and although not significant, there were nearly significant increases in total femur density ($P=0.0722$) and total area ($P=0.0854$) of the female chicks as well (Table 4-3); no

differences in femur weight or length due to sex were observed at 10 d (Table 4-3). At 28 d there were no differences in any BMD measure between the sexes ($P>0.05$; Table 4-3), however total and cortical areas were greater in the male broilers ($P<0.02$), in addition the males also had a greater femur weight ($P<0.0001$) and a nearly significant increase in femur length ($P=0.0620$) than the female broilers (Table 4-3). Femur cross-sectional total, cortical and trabecular areas as well as bone weight were greater for the male birds at 41 d of age, whereas there were no difference between the sexes in any BMD measures or femur length (Table 4-3). Similar results were demonstrated by Yalcin et al. (2001), who found a significant sex effect on bone weight and strength after 16 d of age. In that study, male broilers had a greater bone weight from 16 to 48 d of age, and a greater bone breaking strength at 32 d but not at 48 d. Similar to the results of the current study, Yalcin et al. (2001) found no difference in BMD, measured as bone ash divided by bone volume, between males and females until 48 d of age, when the female BMD was greater than that of the males. One reason male birds might have greater bone areas and strengths than the females may that in the selection process for growth and muscle mass, a stronger frame of the male broilers is also selected. This is supported by the findings of Williams et al. (2000), who reported that fast-growing birds had a greater cortical bone thickness and more rapid bone formation than a slower growing strain. The greater BW also contributes to the greater bone area. When BW was used as a covariate, there was no significant differences between the male and female birds with regards to total or trabecular area ($P=0.14$ and 0.98; respectively) but the male birds had a trend towards a greater cortical area at 28 d ($P=0.07$). In addition, there was no significant difference in

total, cortical area or trabecular area at 41 d (P=0.66, 0.31 and 0.49; respectively) between the male and female birds (data not shown).

There was a treatment by sex interaction for femur length for the Control treatment at 41 d. Among females, those fed the 25D and 25D Early treatments had femur lengths which were significantly shorter than the femurs from those fed the Control treatment; differences due to treatment among the males were not significant (data not shown). This indicates that 25-OH D_3 had a different effect on the male and female birds that requires further investigation.

4.3.4. *Male Broiler Carcass Traits at Processing*

The male birds from the 25D treatment group had the greatest live BW at processing and eviscerated carcass weight (Table 4-5). This is in agreement with the data for the entire population reported in Table 4-4. Similarly, absolute weights of the pectoralis major, wings and drums were greater for the birds from the 25D treatment group than the birds from the Control and 25D Late treatment groups (Table 4-5). In addition, the eviscerated carcass yield as a percentage of live BW was greatest for the birds from the 25D and 25D Early treatment groups (Table 4-5). Given the higher BW in this group, these results are not surprising. However, birds from the 25D treatment also had a greater percentage of pectoralis major muscle than the 25D Early and Late treatments although not different than the Control group (Table 4-5). The birds from the 25D Early treatment group had the greatest percentage of pectoralis minor although not different than the control (Table 4-5). The 25D Early treatment group also yielded the greatest percentage of wings, greater than both the control and 25D treatments (Table 4-5). In addition, the birds from the 25D treatment group had the lowest percentage of

thighs than all the other treatments, while the control had the greatest (Table 4-5). There was no treatment difference in the percentage of drum yield (Table 4-5). Yarger et al. (1995b) also reported that dietary 25-OH D_3 increased breast muscle yield, although not consistently and in only a few of the studies these authors conducted. The studies by Yarger et al (1995b) were carried out under similar dietary and environmental conditions designed to examine the effect of dietary 25-OH D_3 either at similar dietary vitamin D_3 activity as vitamin D_3, or increasing levels of both 25-OH D_3 and vitamin D3. The greater BW and gain of the birds fed the 25-OH D_3 in turn could result in a greater rate of breast muscle deposition. Genetically fast-growing strains have a greater percentage of breast muscle yield than slow growing strains (Fanatico, et al., 2005). This is supported by broiler growth dynamic research which demonstrates that as the birds get bigger, the rate of growth of the breast muscle also increases (Gous, et al., 1999; Zuidhof, 2005). Therefore, if 25-OH D_3 increases growth of the birds, the effect on breast muscle yield can be explained by the phenomenon of allometric growth.

Overall, 25-OH D_3 fed throughout the entire production period or from 0 to 28 d, was able to increase plasma 25-OH D_3 and resulted in a more efficient growth during the grower period (10 to 28 d), improved BMD and area, bone strength and increase breast muscle yield. Although fed at similar levels of vitamin D_3 activity, dietary 25-OH D_3 was able to improve important broiler production parameters. Therefore, 25-OH D_3, may confer additional benefits as compared to vitamin D_3, fed at the same level, especially when available to the bird during the first 28 d post-hatch or throughout the entire production period. However, no benefits were found with the addition of 25-OH D_3 during the later part of the production cycle (from 28 to 41 d).

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Table 4-1. Calculated and analyzed nutrient content of starter, grower and finisher rations

	Starter 0-10 d	Grower 11-28 d	Finisher 29-41 d
Calculated composition			
ME (kcal/kg)	3,130	3,150	3,200
CP (%)	23.2	21.8	20.0
Ca (%)	1.10	1.00	0.90
Total P	0.82	0.80	0.76
aP (%)	0.55	0.50	0.47
Vitamin D_3[1] (IU/kg)	3,000	3,000	3,000
25-OH-D_3[2] (µg/kg)	69	69	69
Analyzed composition (as fed)			
Vitamin D diets			
CP (%)	24.8	24.3	22.1
Ca (%)	1.21	1.08	0.86
Total P (%)	0.79	0.70	0.67
25-OH D_3 diets			
CP (%)	24.9	24.0	21.8
Ca (%)	1.16	1.10	0.77
Total P (%)	0.80	0.72	0.65

[1]Added to feed as Rovimix D_3 500, DSM Nutritional Products (500,000 IU/kg of premix)

[2]Added to feed as Rovimix HyD, DSM Nutritional Products, (138,000 µg of 25-OH D_3/kg of premix).

Table 4-2. Broiler growth and production traits during the starter, grower and finisher periods

		BW (g/bird)				Gain (g/bird/d)			Feed consumption (g/bird/d)			Feed Conversion			
		0 d	10 d	28 d	41 d	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher	Overall
Diet	n														
Control[1]	8	44	205	1182[b]	2234[b]	16.0	53.3[b]	77.9	21.8	81.0[b]	154.7[b]	1.36	1.52	1.99	1.72
25D[2]	8	44	208	1213[a]	2302[a]	16.3	55.0[a]	79.2	22.0	82.9[a]	160.1[a]	1.35	1.51	2.02	1.73
25D Early[3]	8	-	-	-	2272[ab]	-	-	77.5	-	-	156.0[b]	-	-	2.01	1.72
25D Late[4]	8	-	-	-	2234[b]	-	-	77.3	-	-	155.0[b]	-	-	2.01	1.73
SEM		0.1	1.7	7.0	15.9	0.16	0.50	0.88	0.20	0.48	1.42	0.01	0.005	0.01	0.005
Sex															
Female	16	43[b]	209[a]	1153[b]	2129[b]	16.4[a]	51.9[b]	71.9[b]	21.9	79.6[b]	146.6[b]	1.34[a]	1.53[b]	2.04[b]	1.74[a]
Male	16	44[a]	204[b]	1241[a]	2392[a]	15.9[b]	56.4[a]	84.1[a]	21.9	84.3[a]	166.3[a]	1.38[b]	1.50[a]	1.98[a]	1.71[b]
SEM		0.1	1.6	7.4	11.3	0.15	0.38	0.62	0.21	0.50	1.00	0.010	0.005	0.008	0.004
ANOVA	DF	**Probabilities**													
Diet	3	0.8249	0.3404	0.0045	0.0155	0.3028	0.0018	0.4456	0.4514	0.0129	0.0475	0.5789	0.0619	0.2053	0.5657
Sex	1	0.0286	0.0355	<0.0001	<0.0001	0.0303	<0.0001	<0.0001	0.8907	<0.0001	<0.0001	0.0109	<0.0001	<0.0001	<0.0001
Diet*Sex	3	0.9474	0.9948	0.1620	0.6900	0.9978	0.0985	0.5399	0.1909	0.0667	0.2985	0.1386	0.8016	0.9940	0.4098

[ab]Means within the same column and main effect with different subscripts are significantly different (P<0.05).

[1]Birds fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[2]Birds fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[3]Birds fed the 25-OH D_3 diet from 0 to 28 d of age, and the Control diet from 29 to 41 d of age.

[4]Birds fed the Control diet from 0 to 28 d of age, and the 25-OH D_3 diet from 29 to 41 d of age.

Table 4-3. Effect of sex and dietary vitamin D source on femur quality of broilers from 0 – to 41 d[1]

	Femur Density (mg/cm³)			Femur Cross-Sectional Area (mm²)			Femur Weight (g)	Femur Length (cm)
	Total	Cortical	Trabecular	Total	Cortical	Trabecular		
0 d								
Sex								
Female	346.1	586.1	151.2	2.71[a]	0.82	1.26	0.242	2.4[a]
Male	327.8	590.6	121.5	2.49[b]	0.80	1.25	0.218	2.3[b]
SEM	15.66	5.96	14.39	0.07	0.05	0.05	0.010	0.03
ANOVA	Probabilities							
Sex	0.4185	0.5990	0.1627	0.0149	0.8184	0.9687	0.1080	0.0129
Treatment	**10 d**							
Control[2]	508.0[b]	768.5[b]	125.3	8.54	4.51[b]	2.62	1.25	3.72
25D[3]	540.4[a]	792.4[a]	120.6	9.00	5.01[a]	2.68	1.33	3.77
SEM	6.87	5.96	1.71	0.22	0.16	0.09	0.04	0.04
Sex								
Female	533.1	781.0	124.45	9.04	5.09[a]	2.60	1.28	3.76
Male	515.3	779.9	121.40	8.49	4.42[b]	2.70	1.30	3.74
SEM	6.87	5.96	1.71	0.22	0.16	0.09	0.04	0.04
ANOVA	Probabilities							
Treatment	0.0015	0.0065	0.0570	0.1414	0.0372	0.6110	0.1705	0.3662
Sex	0.0722	0.8984	0.2114	0.0854	0.0055	0.4961	0.7367	0.7626
Treatment*Sex	0.9852	0.3654	0.2160	0.0800	0.3271	0.2136	0.0988	0.2534
Treatment	**28 d**							
Control	534.1[b]	845.6[b]	116.7	42.47	23.20	15.65[a]	7.41	6.50
25D	590.2[a]	868.1[a]	109.6	40.07	24.17	12.53[b]	7.31	6.47
SEM	10.62	5.93	3.09	0.99	0.53	0.59	0.14	0.05
Sex								
Female	564.2	865.3	109.7	38.98[b]	22.23[b]	13.65	6.80[b]	6.42
Male	559.8	848.9	116.8	43.69[a]	25.19[a]	14.62	7.92[a]	6.56
SEM	10.62	5.93	3.10	0.99	0.54	0.60	0.14	0.05
ANOVA	Probabilities							
Treatment	0.0005	0.0100	0.1129	0.0934	0.2047	0.0005	0.6192	0.6815
Sex	0.7526	0.0640	0.1104	0.0011	0.0002	0.2158	<0.0001	0.0620
Treatment*Sex	0.3768	0.4964	0.0984	0.9543	0.6740	0.7467	0.9992	0.9261
Treatment	**41 d**							
Control	468.7	908.7	79.7	63.51	35.16	33.97	13.40	8.38[a]
25D	485.2	909.2	83.1	70.64	32.54	34.02	13.69	8.11[ab]
25D Early[4]	483.4	901.8	83.0	70.49	32.51	33.92	13.33	7.91[b]
25D Late[5]	482.7	904.4	82.0	65.66	34.05	32.92	13.45	8.14[ab]
SEM	9.93	7.58	3.00	3.19	2.46	1.71	0.41	0.11
Sex								
Female	476.5	909.5	80.3	61.66[b]	31.03[b]	31.55[b]	12.00[b]	8.07
Male	483.5	902.5	83.7	73.49[a]	36.10[a]	36.80[a]	14.94[a]	8.20
SEM	7.02	5.36	2.12	2.26	1.74	1.21	0.29	0.08
ANOVA	Probabilities							
Treatment	0.6254	0.8832	0.8454	0.3018	0.8453	0.9624	0.9353	0.0289
Sex	0.4815	0.3605	0.2628	0.0005	0.0439	0.0143	<0.0001	0.2193
Treatment*Sex	0.3464	0.6611	0.8102	0.9695	0.8758	0.7280	0.0576	0.0356

[ab]Means within the same column and main effect at each age with different subscripts are significantly different (P<0.05)

[1]At d 0, n = 10 male birds and 10 female birds. At 10 and 28 d, n = 32 birds per treatment (16 male and 16 female). At d 41, n = 16 for each of the 4 dietary treatments (8 male and 8 female);

[2]Birds fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41;

[3]Birds fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 0 to 41 d;

[4]Birds fed the 25-OH D_3 diet from 0 to 28 d of age, and the Control diet from 29 to 41 d of age;

[5]Birds fed the Control diet from 0 to 28 d of age, and the 25-OH D_3 diet from 29 to 41 d of age.

Table 4-4. Effect of dietary vitamin D source on femur quality of male broilers processed at 41d

	n	Femur Density (mg/cm^3)			Femur Cross-sectional Area (mm^2)			Femur Weight (g)	Femur Length (cm)	Bone Breaking Strength[1] (KgF)
		Total	Cortical	Trabecular	Total	Cortical	Trabecular			
Treatment										
Control[2]	51	457.3[b]	900.9	80.6	77.38	33.82[b]	39.29	15.18	8.3	34.27[b]
25D[3]	48	482.7[a]	915.8	82.1	80.14	36.66[a]	39.03	15.63	8.3	40.62[a]
25D Early[4]	49	481.8[a]	914.6	83.4	77.13	35.41[ab]	37.61	15.21	8.2	40.15[a]
25D Late[5]	49	465.3[b]	908.8	78.6	75.84	33.57[b]	38.06	15.41	8.3	33.89[b]
SE		5.66	4.17	2.25	1.45	0.70	0.93	0.27	0.06	1.44
ANOVA		Probabilities								
Treatment		0.0032	0.0516	0.4992	0.2226	0.0077	0.5480	0.6406	0.8404	0.0006
BW as Covariate										
Treatment										
Control	51	457.8[b]	898.3[b]	81.6	77.39	34.40	39.62	15.29	8.3	36.63[ab]
25D	48	482.1[a]	915.3[a]	81.88	78.83	36.04	38.39	15.38	8.3	39.94[a]
25D Early	49	483.8[a]	914.6[a]	83.4	77.15	35.41	37.62	15.21	8.2	39.92[a]
25D Late	49	464.8[b]	908.4[ab]	79.0	76.35	33.76	38.35	15.51	8.3	33.95[b]
SE		5.85	4.23	2.32	1.17	0.60	0.82	0.21	0.06	1.44
ANOVA		Probabilities								
Treatment		0.0044	0.0241	0.6029	0.4414	0.0526	0.3954	0.7842	0.7481	0.0323
BW		0.6531	0.6314	0.4922	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0069

[ab]Means within the same column and main effect at each age with different subscripts are significantly different ($P<0.05$)

[1] N for bone breaking strength = 20 per treatment

[2]Birds fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[3]Birds fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[4]Birds fed the 25-OH D_3 diet from 0 to 28 d of age, and the Control diet from 29 to 41 d of age.

[5]Birds fed the Control diet from 0 to 28 d of age, and the 25-OH D_3 diet from 29 to 41 d of age.

Table 4-5. Effect of vitamin D source on carcass traits of 42 d male broilers

		BW (g/bird)		P. Major	P. Minor	Wings	Thighs	Drums
		Live	Eviscerated					
				Absolute weight (g)				
Diet	n							
Control[1]	77	2386.7^{b}	1596.24^{b}	343.14^{b}	81.63	198.67^{c}	292.55	247.87^{b}
25D[2]	77	2501.9^{a}	1683.54^{a}	367.05^{a}	84.07	209.00^{a}	292.29	263.28^{a}
25D Early[3]	78	2413.9^{b}	1630.54^{b}	340.39^{b}	85.14	206.00^{ab}	292.06	256.08^{ab}
25D Late[4]	79	2388.4^{b}	1595.30^{b}	333.36^{b}	81.11	200.63^{bc}	287.85	248.86^{b}
SEM		27.62	18.721	5.045	1.230	2.081	3.922	3.091
ANOVA	DF			Probabilities				
Diet	3	0.0081	0.0023	<0.0001	0.0622	0.0015	0.8020	0.0012
			% of Live BW	**% of Eviscerated Carcass Weight**				
Diet	n							
Control	77	-	66.83^{b}	21.48^{a}	5.11^{ab}	12.47^{b}	18.33^{a}	15.54
25D	77	-	67.50^{a}	21.77^{a}	4.98^{c}	12.44^{b}	17.37^{c}	15.66
25D Early	78	-	67.51^{a}	20.88^{b}	5.22^{a}	12.68^{a}	17.92^{b}	15.72
25D Late	79	-	66.74^{b}	20.90^{b}	5.08^{bc}	12.60^{ab}	18.07^{ab}	15.60
SEM		-	0.155	0.162	0.045	0.063	0.136	0.090
ANOVA	DF			Probabilities				
Diet	3	-	0.0001	<0.0001	0.0020	0.0275	<0.0001	0.5386

$^{a-c}$ Means within the same column and variable with no common postscripts are significantly different ($P<0.05$)

[1]Birds fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[2]Birds fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from d0 to d 41

[3]Birds fed the 25-OH D_3 diet from 0 to 28 d of age, and the Control diet from 29 to 41 d of age.

[4]Birds fed the Control diet from 0 to 28 d of age, and the 25-OH D_3 diet from 29 to 41 d of age.

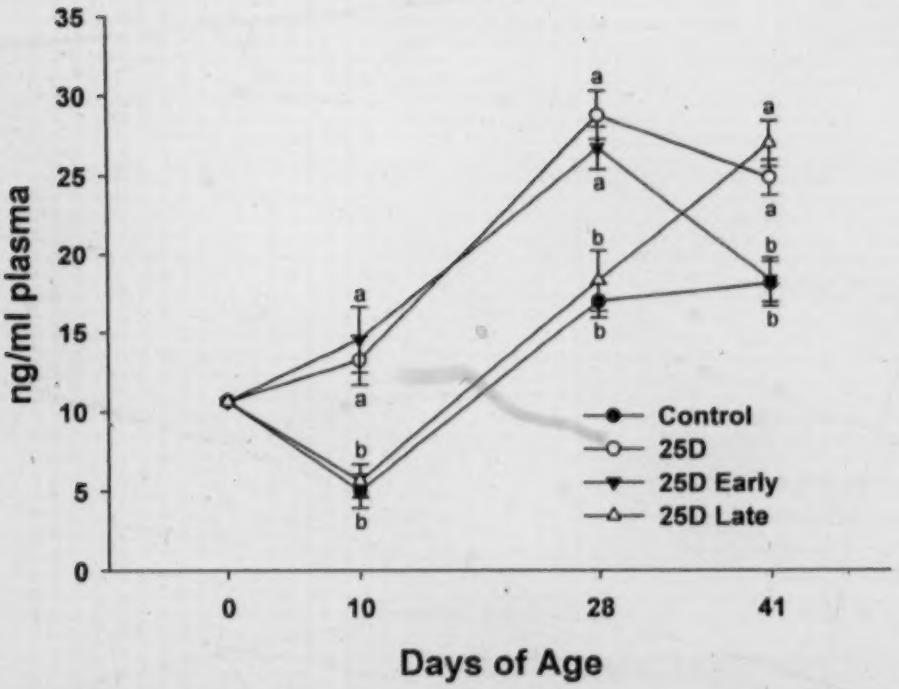

Figure 4-1. Effect of dietary vitamin D_3 source on broiler plasma 25-OH D_3 from hatch to 41 d of age.
Control = Birds fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 0 to 41 d; 25D = Birds fed a diet containing 69 µg/kg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 0 to 41 d; 25D Early = Birds fed the 25D diet from 0 to 28 d, and the Control diet from 29 to 41 d; 25D Late = Birds fed the Control diet from 0 to 28 d of age, and the 25D diet from 29 to 41 d of age. Means at the same age, with different letters are significantly different ($P<0.05$).

CHAPTER 5: The Effect of Dietary Vitamin D Source on Broiler Immune Responses and Bone Quality

5.1. INTRODUCTION

Vitamin D is crucial in Ca homeostasis so that Ca can be available for bone formation and other critical functions. The vitamin D metabolite, $1,25(OH)_2D_3$, is involved in the deposition of skeletal minerals, of which Ca comprises the greatest amount, as well as Ca resorption from the bone tissues when plasma Ca levels are low (DeLuca, 2004).

There are 2 major arms of the immune system, the innate and the acquired. The innate immune system is the first line of defense, the cells of this system work to recognize, phagocytise (engulf bacteria) and, using non-specific mechanisms, kill the invading pathogen as well as to signal the acquired immune response (Zekarias, et al., 2002). The main cellular players in acquired immunity are the lymphocytes, which are divided into B and T lymphocytes. The B-cells recognize specific antigens and make antibodies to that specific antigen (Sharma, 1997). The T-cells, are divided into helper T-cells (CD4), and cytotoxic/suppressor T-cells (CD8), the former which work to initiate specific immune responses to particular antigen recognition and the latter suppresses immune responses (Sharma, 1997).

Nutrition plays a major role in immune function and the overall health of the bird. Nutritional deficiencies often lead to immune depression, making the bird more susceptible to diseases and infections (Kidd, et al., 2001). Vitamin D is involved in various aspects of the immune system. Human and rodent research has linked it to

various aspects of both acquired and innate immune function, including, but not limited to; promoting monocyte maturation, neutrophil chemotaxis, enhancing phagocytic and bactericidal/tumorcidal capability of leukocytes, enhancing antigen presentation, inhibiting Interlukin-2, suppressing inflammatory T cell response and inhibiting lymphocyte proliferation (Cohen and Gray, 1984; Gray and Cohen, 1985; Manolagas, et al., 1985; Reinhardt and Hustmyer, 1987; Manolagas, et al., 1989; Binder, et al., 1999; Brown, et al., 1999; Deluca and Cantorna, 2001; Griffin, et al., 2003; Cantorna, et al., 2004; Gomme and Bertolini, 2004; Gombart, et al., 2005). Reports on the involvement of vitamin D in the immune function of poultry species have shown a depressed cellular immune response in vitamin D-deficient chicks (Aslam, et al., 1998). However there was no difference between dietary vitamin D and 25-OH D_3 in *ex vivo* 21 d macrophage nitric oxide production and cytotoxicity as well as 35 d cutaneous basophil hypersensitivity at adequate levels of each (Fritts, et al., 2004).

In mammals, the active vitamin D metabolite, $1,25(OH)_2D_3$ is involved in the cellular immune response. The vitamin D receptor has been isolated from activated T- and B-lymphocytes (Provvedini, et al., 1983; Manolagas, et al., 1989). Most mammalian research indicates that $1,25(OH)_2D_3$ can have either proliferative or antiproliferative effects on T cells, depending on which immunological pathways are activated (Manolagas, et al., 1989). The rate of B-cell proliferation as well as activity is decreased in the presence of $1,25(OH)_2D_3$ (Brown, et al., 1999). In addition, immunoglobulin production has been shown to be inhibited in the presence of $1,25(OH)_2D_3$ in vitro (Provvedini, et al., 1986).

Twenty-five-OH D_3 is the most abundant circulating form of vitamin D (Haussler and Rasmussen, 1972). Due to the commercial availability of 25-OH D_3 to the poultry industry (HyD™, DSM Nutritional Products, Parsippany, NJ), the opportunity exists to improve the vitamin D status of the chick by feeding dietary 25-OH D_3. This product has shown no signs of toxicity at up to 10 times the recommended feeding concentration of 69 µg/kg (Yarger, et al., 1995a). Levels of 25-OH D_3 are not as tightly regulated within the body as its more potent and active metabolite, $1,25(OH)_2D_3$ (Combs, 1992). Providing the chick with a dietary source of 25-OH D_3 may allow for more of this substrate to be available for the conversion to $1,25(OH)_2D_3$ and the potential to enhance the functions that vitamin D metabolites serve within the body. Previous studies have shown dietary 25-OH D_3 to increase BW (Yarger, et al., 1995b; Mitchell, et al., 1997; Aburto, et al., 1998), and to improve feed conversion efficiency in broilers (Yarger, et al., 1995b) in comparison to vitamin D_3 when fed at similar vitamin D activity levels. The addition of 25-OH D_3 to the diet may therefore offer a greater potential over vitamin D_3 in improving broiler performance and bone integrity.

The effects of 25-OH D_3 on broiler BW and FCE, as well as the potential immunological effects of dietary 25-OH D_3 require further investigation. Therefore the objectives to the present study were to examine the effects of dietary 25-OH D_3, alone or in combination with vitamin D_3 on broiler chicken performance, bone development, the inflammatory response and antibody production in broilers.

5.2. MATERIALS AND METHODS

5.2.1. *Dietary Treatments*

Experimental diets were formulated to meet or exceed current NRC recommendations (National Research Council, 1994) and were based on the primary breeder recommendations for Cobb mixed-sex broilers (Cobb-Vantress Inc, 2004). The birds were fed a crumbled starter ration (23.5 % CP, 3,134 kcal/kg ME, 1.10 % Ca, and 0.55% available P) from 0 to 10 d of age. The grower (21.8 % CP, 3,150 kcal/kg ME, 1.00% Ca, and 0.50% available P; fed from 11 to 28 d of age) and finisher (20% CP, 3,200 kcal/kg ME, 0.90% Ca, and 0.47% available P; fed from 29 to 42 d of age) rations were pelleted. All diets were wheat-based and supplemented with a commercial arabinoxylanase enzyme (Avizyme 1302, Danisco Animal Nutrition, Marlborough, UK) at the manufacturer's recommended level. One series of experimental diets were formulated to contain vitamin D_3 at 2,500 IU/kg of feed plus 25-OH D_3 added at 0% (D; Control), 25% (D+ 25HD), 50% (D+ 50HD), 100% (D+100HD) or 150% (D+150HD) of manufacturer's recommended levels (0, 17.25, 34.5, 69 or 103.5 µg/kg diet, respectively). A second series of diets contained the same levels of 25-OH D_3 in the absence of vitamin D_3 (25HD, 50HD, 100HD, 150HD; respectively), for a total of 9 diets.

5.2.2. *Broiler Production*

This experimental protocol was approved by the University of Alberta's Faculty Animal Policy and Welfare Committee under the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993). Day-old, Cobb 500 (N= 720) broiler chicks were wing-banded and randomly allocated to 72 Specht pullet rearing cages (n=10 per cage; 0.63 m^2 per bird; 8 cages per dietary treatment). Birds were raised in a light-tight barn with incandescent lighting provided 23 $h \cdot day^{-1}$ and had *ad libitum*

access to feed and water (nipple drinkers). A 3-phase feeding program with a starter (0 to 10 d), grower (11 to 28 d) and finisher (29 to 42 d) period was used. At days 0, 10, 28 and 42, BW on a pen basis was obtained and feed consumption measured for the starter, grower and finisher phases. Mortality-corrected feed conversion ratios (g feed/g gain) were calculated.

5.2.3. *Inflammatory Immune Response*

At 11 and 13 d of age, 2 birds per pen (n=16 per dietary treatment) were selected at random, wing-band numbers were recorded, and each bird was weighed and injected with 3 ml of a 100 µg/ml solution of *Salmonella typhimurium* lipopolysaccharide (LPS) to simulate an infectious challenge (Korver, et al., 1998). Within each pen, 2 additional chicks (n=16 per dietary treatment) were chosen at random to serve as non-injected controls. Previous research has shown no difference between non-injected and saline injected controls (Benson, et al., 1993). The wingband numbers were recorded and the birds were weighed. On day 14 of age, individual body weights of all 4 of the identified birds per pen were determined. In addition, at 14 d the right femur of 1 injected and 1 control bird per pen was removed and stored at -20 C until further analysis for bone mineral density (BMD) and breaking strength. At 42 d of age, the right femur of the remaining LPS injected bird was collected and stored at -20 C until further analysis.

5.2.4. *Antibody Titer Response*

At 28 and 35 days of age, 2 additional birds per pen (n=16 per dietary treatment) were selected at random and vaccinated with a 4-way commercial vaccine for infectious

bursal disease virus, Newcastle disease, infectious bronchitis and avian reovirus (Breedervac-IV, Intervet Inc., Delaware, USA). The selected birds were bled by brachial venipunture at days 35 and 41 to assess primary and secondary antibody responses. Newcastle disease virus antibody titers were measured using an ELISA kit (Breedervac-IV, Intervet Inc., Delaware, USA).

5.2.5. *Bone Mineral Density and Cross-sectional Area*

Bone mineral density and cross-sectional area analysis were performed by quantitative computed tomography using a Stratec Norland XCT (XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA) scanner with a 50kV x-ray tube using a similar procedure as outlined in Riczu et al (2004). The scanner was calibrated daily prior using a multi-slice standard phantom. Each bone scan was preformed at the mid-shaft of each femur. Soft tissue surrounding the bone was differentiated from the outer cortical bone surface, an inner threshold level was set at 400 mg/cm^3 to separate cortical and subcortical from trabecular bone and a threshold of 500 mg/cm^3 was used to separate cortical from sub-cortical bone regions.

5.2.6. *Bone Breaking Strength*

Breaking strength analysis was performed using a slightly modified version of the method described in Fleming et al. (1998). An Instron Materials Tester (Model 4411, Instron Corp., Canton, Ma, USA) with Automated Materials Test System software version 8.09, a standard 50 kg load cell, and a modified sheer plate (8 cm in length and 1 mm in width) were used. A distance of 3 cm for the 14 d bones and 6 cm for the 42 d bones was set between 2 fixed points supporting the bone, and a crosshead speed of 100

mm/min was held constant throughout each measurement. Bone breaking strength was measured in Kg of Force (Kg F) required to reach the break point.

5.2.7. *Statistical Analysis*

The pen was the experimental unit for production data (n=8 per diet), whereas the individual bird was the experimental unit for the bone, LPS challenge (both bone (n=72 per treatment) and BW data (n=144 per treatment)) and antibody titer data. Broiler production data and antibody titers were analyzed as a 1-way analysis of variance with diet as the main factor. For the inflammatory immune response, data were analyzed as a 9 X 2 factorial with 9 dietary treatments and 2 LPS treatments. All data were analyzed using the Mixed model analysis of SAS and significance was assessed at a probability of $P < 0.05$ (SAS Institute, 1999). Means were compared using the LSmeans comparisons procedure of SAS (SAS Institute, 1999).

5.3. RESULTS AND DISCUSSION

5.3.1. *Effect of Vitamin D Source and Level on Broiler Production*

There were no dietary treatment effects on BW, BW gain, feed consumption or feed conversion efficiency during the starter, grower or finisher periods, or over the entire course of the experiment (Table 5-1). This is in contrast to previous studies conducted by our group comparing supplementation of broiler diets with vitamin D and 25-OH D_3 in which increased broiler BW, BW gain were observed, particularly in the grower period (Chapter 4; Table 4-4). These results are also not in agreement with those of Yarger et al. (1995b), who reported that dietary 25-OH D_3, increased final BW when comparing dietary 25-OH D_3 and vitamin D_3 across a range of levels of vitamin D activity, including

the levels used in the current study. Results similar to Yarger et al. (1995b) have also been reported in other studies in which 16 d broiler BW were greater in broilers either supplemented with > 5μg/kg of 25-OH D_3 either alone or in addition to dietary vitamin D_3 when compared with vitamin D_3 alone (Mitchell, et al., 1997; Aburto, et al., 1998). However, Bar et al. (2003), found broiler BW at 7 and 22 d of age were increased by dietary 25-OH D_3, but only when the diet was slightly restricted in P. This effect was not observed when diets sufficient in P were fed (Bar, et al., 2003), as the level of vitamin D required by the bird may be altered by the levels of other nutrients such as Ca and P in the diet (Whitehead, et al., 2004). In the current study, Ca and P were not limiting and therefore would be in agreement with those results obtain by Bar et al. (2003) when Ca and P were not restricted. The likely reasons for the differing results than those reported in the previous broiler study (Chapter 4), could be due to the different strain of birds (Ross vs Cobb) or the environment in which birds were reared (floor vs cage). In humans, the vitamin D receptor (VDR) has been found to have several genetic variations (polymorphisms) that might influence functions in which vitamin D is involved (Uitterlinden, et al., 2004; Valdivielso and Fernandez, 2006). Recently, polymorphisms of the chicken VDR associated with differences in bone traits have been reported (Bennett, et al., 2006). These polymorphisms may therefore influence the effect of the dietary treatments in the current research and may explain the differing results between the studies in Chapter 4 and the current chapter.

5.3.2. *Effect of Vitamin D Source and Level on the Effects of LPS Injection*

There was no effect of diet on BW of the broilers at 11, 13 or 14 d of age as well as BW gain from 11 to 13 d (Table 5-2). However, the birds on D, D+50HD and the 100HD all had the lowest BW gain from 13-14 d of age, with birds on all the other treatments having greater BW gain than these 3 treatments ($P<0.02$; Table 5-2). These results are not what were expected; both the D (2,500 IU vitamin D_3) and the current industry recommended level of 25-OH D_3 (69 μg/kg) treatments (100HD) resulted in a lower growth rate compared to the other treatments (Table 5-2). There were no differences due to dietary treatment on overall body weight gain from 11 to 14 d of age (Table 5-2). Therefore, the reduction in BW gain from 13 to 14 d did not have a significant effect on total gain from 11 to 14 d (Table 5-2).

The LPS-injected birds had reduced BW ($P = 0.007$) in the 2 days following the first LPS injection as a result of a slower rate of BW gain ($P = 0.005$) than the non-injected birds during this time (Table 5-2). In addition, BW at 14 d was also greater for the Control birds than the LPS injected birds ($P = 0.002$), with the Control birds having a greater rate of gain from 13 to 14 d of age ($P = 0.0005$; Table 5-2). Overall, the control birds had a greater rate of BW gain from 11 to 14 d than the LPS injected birds ($P<0.0001$; Table 5-1). These results were expected, as the inflammatory response to the LPS injection diverts nutrients away from growth and towards the acute phase response resulting in muscle degradation and depressed growth rates (Klasing, 1998; Barnes, et al., 2002; Mireles, et al., 2005). These results are similar to those reported by Mireles et al. (2005), who also reported a reduction in broiler growth over a short period of time (24 hr) when broilers were injected with LPS. As there were no dietary by injection treatment interaction effects, dietary 25-OH D_3 fed at any level or in conjunction with vitamin D_3

did not alter the effect of the LPS injections on BW gain from 11 to 14 d of age (Table 5-2). This may be an effect of the short duration of the LPS challenge. To the authors' knowledge, no reports have addressed the effect of dietary vitamin D sources on BW gain during an inflammatory immune response.

Femur total and trabecular BMD, total, trabecular and cortical areas, bone weight, bone length and bone breaking strength of broilers at 14 d (4 d after LPS injection) were not different among any of the dietary treatments (Table 5-3). However, femur cortical BMD of broilers at 14 d was greatest for those birds which received either D+100HD or 150HD ($P = 0.0084$; Table 5-3). Across all dietary treatments, LPS injection resulted in a lower total bone area ($P = 0.0017$) and bone weight ($P = 0.0035$), but greater total ($P = 0.0232$) and cortical (0.0064) BMD as well as a nearly significant increase in bone length ($P = 0.0805$; Table 5-3). However, there was no effect of LPS treatment on bone breaking strength. There was a significant interaction effect of diet and treatment on trabecular area at 14d of age ($P = 0.0060$; Table 5-3). Mireles et al. (2005), found bone weight and bone breaking strength to be reduced 3 d after a single injection of LPS. These authors also reported that although BW and bone breaking strength were positively correlated, bone breaking strength was also dependent on the level of LPS injected, with decreased breaking strength associated with increasing amounts of LPS. In addition, bone breaking strength was found to be a sensitive measure of the intensity of an acute phase response. However, in the current study cortical BMD appeared to be a more sensitive measure than bone breaking strength for observing changes in bone structure during an inflammatory immune response. Overall, dietary 25-OH D_3 at the recommended level, 69 μg/kg of feed, in addition to vitamin D_3 or at 150% (103.5 μg/kg

of feed) of the recommended level on its own helped to reduce the effect of an inflammatory challenge on bone catabolism. There were no dietary treatment effects on bone weight or length (Table 5-3). Again, to the authors' knowledge, there are no previous reports comparing the effects of dietary vitamin D source on bone density and strength during an inflammatory immune response.

The birds that were injected with LPS had the lowest total and trabecular bone area (Table 5-3). However, these birds had a greater total and cortical BMD (Table 5-3). The reason for this could be that the BW of the non-injected broilers was greater than the LPS-injected broilers (Table 5-3), and as the birds grow so does the width of the bone (or cross-sectional bone area) (Williams, et al., 2000). However, the fast growth rate of modern broiler strains has resulted in a more porous bone formation to provide as much strength as possible given the limited ability and time to form cortical bone (Williams, et al., 2000). In the current study, the fact that the growth rate of the LPS injected birds was decreased (Table 5-2) could have resulted in less porous bone formation which in turn resulted in the greater cortical BMD of the LPS injected broilers (Table 5-3). At 14 d there were no differences in bone breaking strength or length between LPS-injected and non-injected controls, whereas bone weight was greatest in the non-injected birds (Table 5-3).

Femur BMD and areas, bone weight and length from LPS-injected birds were not different among the dietary treatments at 42 d of age (Table 5-4), indicating no lasting effect of the LPS injections at 2 wk of age on bone structure. However, among the birds injected with LPS at 11 and 13 d of age, bone breaking strength at 42 d was nearly greatest for the birds fed the 100HD diet ($P<0.08$; Table 5-4). To the author's

knowledge, no previous studies have looked at the effect of 25-OH D_3 following an inflammatory challenge. Previous work in our lab has also shown dietary 25-OH D_3 to increase bone breaking strength in the absence of an experimental inflammatory challenge (Chapter 4; Table 4-4). Dietary 25-OH D_3 has been found to have a positive effect on bone ash and the incidence and severity of tibial dyschrondroplasia of broilers (Applegate, et al., 2003; Fritts and Waldroup, 2003). Bar et al. (2003) found no difference in the amount of bone ash from broilers fed either dietary vitamin D_3 or 25-OH D_3 in a series of experiments designed to examine the use of 25-OH D_3 either as a complete replacement of dietary vitamin D_3, or in addition to dietary vitamin D_3 (at both adequate and deficient levels). The single experiment by Bar et al. (2003) which showed 25-OH D_3 supplementation to increase tibia ash, was when dietary Ca and P were slightly restricted. Similarly, Ledwaba and Roberson (2003) found 25-OH D_3 to be effective at reducing the severity of TD when dietary Ca was low. However in the current study, measurements of bone breaking and not bone ash nor individual bone minerals were compared among dietary treatments, and may be the reason for the differences in the conclusions drawn. Even at the lowest level of 25-OH D_3 inclusion (17.25 µg/ kg or 25% of recommended level), the birds still received over 3 times the NRC recommended vitamin D activity.

5.3.3. *Effect of Vitamin D Source and Level on Primary and Secondary Antibody Response*

Primary and secondary antibody responses to Newcastle disease virus were not different among the dietary treatment groups, although these results had a high amount of

variation (Table 5-5). The results of the current study show that dietary 25-OH D_3, alone or in combination with vitamin D_3 did not affect the Ab response to a vaccine in broilers. Similarly, in response to sheep erythrocytes, primary and secondary antibody production was not different between chicks fed vitamin D-sufficient or -deficient diets, showing that antibody immune response is not affected by vitamin D status (Aslam, et al., 1998). Veldman et al. (2000), found that mice B cells do not express the vitamin D receptor at rest, when activated nor in the presence of 1, $25(OH)_2$ D_3.

In summary, in response to a minor bacterial toxin challenge supplementing the diet with 25-OH D_3 reduced the expected decrease in bone catabolism without an effect on BW or ability to respond to a vaccine. Therefore routine supplementation of the diet of chicks with 25-OH D_3 could be beneficial in reducing bone catabolism after a bacterial challenge.

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Table 5-1. Effect of dietary vitamin D source and level of 25-OH vitamin D_3 on broiler growth and production during the starter, grower and finisher periods[1]

	BW (g/bird)				Gain (g/bird/d)			
	0 d	10 d	28 d	42 d	Str[2]	Gwr[3]	Fin[4]	Overall
Diet[5]								
D	47.0	247	1,299	2,491	20.0	60.1	86.1	53.8
D+25HD	47.4	246	1,350	2,511	19.8	62.2	88.0	54.9
D+50HD	47.5	237	1,314	2,419	18.8	60.6	84.7	52.0
D+100HD	46.7	240	1,317	2,462	19.4	61.7	85.0	54.2
D+150HD	46.8	250	1,284	2,408	20.3	59.2	86.0	53.8
25HD	46.5	246	1,326	2,432	19.8	61.9	84.4	54.6
50HD	46.1	232	1,294	2,433	18.6	60.2	87.6	54.3
100HD	45.9	240	1,291	2,397	19.4	59.9	84.1	53.1
150HD	46.8	242	1,308	2,443	19.5	61.0	83.1	53.5
SEM	0.51	5.3	28.3	47.1	0.52	1.24	2.58	0.92
ANOVA				Probabilities				
Diet	0.3208	0.3274	0.7759	0.6647	0.3594	0.6434	0.8962	0.4884
	Feed consumption (g/bird/d)				FCR[6]			
	Str	Gwr	Fin	Overall	Str	Gwr	Fin	Overall
Diet								
D	24.2	86.2	135.8	79.0	1.21	1.44	1.58	1.47
D+25HD	24.1	88.7	139.7	80.8	1.22	1.43	1.59	1.47
D+50HD	23.8	87.5	136.2	79.5	1.27	1.44	1.61	1.49
D+100HD	24.3	88.0	136.3	80.4	1.26	1.43	1.61	1.48
D+150HD	25.0	86.6	137.9	80.4	1.24	1.46	1.60	1.50
25HD	24.2	88.1	136.0	80.8	1.22	1.42	1.61	1.48
50HD	23.1	86.3	137.3	80.6	1.23	1.43	1.57	1.47
100HD	23.8	84.4	133.5	77.7	1.22	1.41	1.59	1.46
150HD	24.4	88.1	135.1	80.2	1.25	1.44	1.63	1.50
SEM	0.50	1.70	3.30	1.31	0.02	0.01	0.03	0.01
ANOVA			Probabilities					
Diet	0.3317	0.6883	0.9568	0.7535	0.4264	0.2407	0.9197	0.4525

[1]n=8 pens per dietary treatment.
[2]Starter phase = 0 to 10 d of age.
[3]Grower phase = 11 to 28 d of age.
[4]Finisher phase = 29 to 42 d of age.
[5]D=2,500 I.U. vit. D_3; D+25HD= 2,500 I.U. vit. D_3 + 17.25 µg 25-OH D_3; D+50HD=2,500 I.U. vit. D_3 + 34.5 µg 25-OH D_3; D+100HD –2,500 I.U. vit. D_3 + 69 µg 25-OH D_3; D+150HD= 2,500 I.U. vit. D_3 + 103.5 µg 25-OH D_3; 25HD= 17.25 µg 25-OH D_3; 50HD= 34.5 µg 25-OH D_3; 100HD= 69 µg 25-OH D_3; 150HD= 103.5 µg 25-OH D_3.
[6]FCR= feed conversion ratio (g of feed/g of gain).

Table 5-2. Effect of dietary vitamin D source and LPS treatment on broiler growth from 11 to 14 d[1]

		— BW (g/bird) —			— Gain (g/bird/d) —		
		11 d	13 d	14 d	Gain 1 (11 -13d)	Gain 2 (13-14 d)	Total Gain (11-14 d)
Diet[2]	**n**						
D	8	247.75	316.87	365.99	34.56	49.11[b]	39.41
D+25HD	8	243.56	308.97	368.64	33.76	54.93[a]	40.81
D+50HD	8	238.56	308.34	362.81	34.83	51.40[b]	40.35
D+100HD	8	247.44	318.72	374.59	35.64	55.88[a]	40.38
D+150HD	8	244.16	311.89	367.49	34.08	55.60[a]	41.26
25HD	8	249.63	316.31	373.38	34.42	57.20[a]	42.01
50HD	8	233.31	296.75	352.07	31.72	55.32[a]	39.59
100HD	8	235.09	300.94	353.39	32.92	52.45[b]	39.43
150HD	8	245.13	307.12	365.47	31.00	58.01[a]	39.97
	SEM	4.98	6.44	7.56	1.14	1.92	1.06
Treatment	**n**						
Control	144	241.91	315.43[a]	372.77[a]	36.75[a]	56.66[a]	43.38[a]
LPS	144	243.56	303.66[b]	356.97[b]	30.57[b]	52.21[b]	37.78[b]
	SEM	2.35	3.04	3.53	0.54	0.89	0.50
ANOVA		**Probabilities**					
Diet		0.2187	0.2381	0.3600	0.0857	0.0179	0.3473
Treatment		0.6189	0.0066	0.0017	0.0001	0.0005	<0.0001
Diet*Treatment		0.5982	0.5147	0.3950	0.6943	0.6822	0.6662

[1]Initial BW at 11 d of age, prior to initiation of treatment (lipopolysaccharide (LPS) injection or control). LPS-injected birds were injected intra-abdominally with 3 ml of a 100 µg/ml solution of LPS at 11 and 13d. Control birds were not injected.

[2]D= 2,500 I.U. vit. D_3; D+25HD= 2,500 I.U. vit. D_3 + 17.25 µg 25-OH D_3; D+50HD=2,500 I.U. vit. D_3 + 34.5 µg 25-OH D_3; D+100HD =2,500 I.U. vit. D_3 + 69 µg 25-OH D_3; D+150HD= 2,500 I.U. vit. D_3 + 103.5 µg 25-OH D_3; 25HD= 17.25 µg 25-OH D_3; 50HD= 34.5 µg 25-OH D_3; 100HD= 69 µg 25-OH D_3; 150HD= 103.5 µg 25-OH D_3.

[a-b] Means within the same column with no common superscripts are significantly different (P<0.05).

Table 5-3. Effect of dietary vitamin D source and LPS treatment on bone quality at 14 d of age[1]

		—— Density (mg/cm³) ——			—— Area (mm²) ——			Bone weight (g)	Bone Length (mm)	Bone Breaking Strength (KgF)
		Total	Cortical	Trabecular	Total	Cortical	Trabecular			
Diet[2]	**N**									
D	8	517.54	745.39[b]	125.14	14.07	7.85	3.94	2.01	4.37	11.00
D+25HD	8	531.21	751.65[b]	131.42	15.54	9.18	4.12	2.27	4.52	12.64
D+50HD	8	522.37	752.69[b]	130.51	13.56	7.74	3.77	2.03	4.42	11.53
D+100HD	8	542.04	775.65[a]	128.25	13.98	8.25	3.76	2.18	4.41	11.05
D+150HD	8	523.53	749.37[b]	132.84	14.97	8.60	4.09	2.13	4.42	12.16
25HD	8	530.53	751.59[b]	135.67	14.32	8.37	3.91	2.05	4.42	11.20
50HD	8	533.04	763.30[ab]	129.32	13.74	8.01	3.76	2.04	4.43	11.52
100HD	8	510.89	752.44[b]	126.12	13.54	7.69	3.99	2.06	4.41	10.90
150HD	8	551.93	776.07[a]	124.14	14.01	8.50	3.71	2.07	4.37	12.27
	SEM	9.20	6.91	4.41	0.52	0.42	0.13	0.08	0.06	0.76
Treatment	**n**									
Control	72	522.20[b]	751.1[b]	130.27	14.75[a]	8.36	3.99[a]	2.18[a]	4.38	11.80
LPS	72	536.35[a]	763.96[a]	128.27	13.64[b]	8.12	3.80[b]	2.01[b]	4.46	11.37
	SEM	4.37	3.26	2.02	0.25	0.20	0.06	0.04	0.03	0.36
ANOVA		**Probabilities**								
Diet		0.0809	0.0084	0.6213	0.1214	0.2406	0.1888	0.3971	0.8561	0.6987
Treatment		0.0232	0.0064	0.4847	0.0017	0.4120	0.0273	0.0035	0.0805	0.4010
Diet*Treatment		0.1467	0.0736	0.1675	0.3396	0.4343	0.0060	0.6652	0.9848	0.7244

[1]Initial BW at 11 d of age, prior to initiation of treatment (lipopolysaccharide (LPS) injection or control). LPS-injected birds were injected intra-abdominally with 3 ml of a 100 µg/ml solution of LPS at 11 and 13d. Control birds were not injected.

[2]The following are the dietary treatments: D= 2,500 I.U. vit. D_3; D+25HD= 2,500 I.U. vit. D_3 + 17.25 µg 25-OH D_3; D+50HD=2,500 I.U. vit. D_3 + 34.5 µg 25-OH D_3; D+100HD –2,500 I.U. vit. D_3 + 69 µg 25-OH D_3; D+150HD= 2,500 I.U. vit. D_3 + 103.5 µg 25-OH D_3; 25HD= 17.25 µg 25-OH D_3; 50HD= 34.5 µg 25-OH D_3; 100HD= 69 µg 25-OH D_3; 150HD= 103.5 µg 25-OH D_3.

[a-b] Means within the same column with no common subscripts are significantly different (P<0.05).

Table 5-4. Effect of dietary vitamin D source on bone quality at 42 d of age from LPS-injected birds[1]

		Density (mg/cm^3)			Area (mm^2)			Bone weight (g)	Bone Length (mm)	Bone Breaking Strength (KgF)
		Total	Cortical	Trabecular	Total	Cortical	Trabecular			
Diet[2]	**n**									
D	8	423.96	883.46	83.74	81.25	32.94	44.83	15.47	8.64	33.66
D+25HD	8	434.20	914.26	81.30	82.29	33.75	45.70	16.12	8.46	34.96
D+50HD	8	422.98	910.19	72.83	85.96	35.51	47.86	17.13	8.65	35.78
D+100HD	8	431.28	903.83	88.31	78.10	31.53	43.91	14.76	8.34	32.33
D+150HD	8	434.10	882.56	88.94	80.84	33.49	44.05	15.17	8.58	36.12
25HD	8	444.09	930.99	73.78	76.29	31.97	41.42	15.43	8.46	35.69
50HD	8	450.23	908.73	80.38	80.30	34.53	42.56	16.07	8.50	38.35
100HD	8	454.15	899.14	80.26	77.42	33.95	40.49	15.81	8.43	42.92
150HD	8	421.50	899.91	77.98	79.42	31.94	44.38	14.98	8.40	35.95
SEM		13.77	12.43	6.49	4.35	1.72	2.82	0.95	0.18	2.20
ANOVA		**Probabilities**								
Diet		0.6567	0.1878	0.6501	0.8916	0.7906	0.7550	0.8033	0.9321	0.0771

[1]Initial BW at 11 d of age, prior to initiation of treatment (lipopolysaccharide (LPS) injection or control). LPS-injected birds were injected intra-abdominally with 3 ml of a 100 µg/ml solution of LPS at 11 and 13d. Control birds were not injected.

[2]D= 2,500 I.U. vit. D_3; D+25HD= 2,500 I.U. vit. D_3 + 17.25 µg 25-OH D_3; D+50HD=2,500 I.U. vit. D_3 + 34.5 µg 25-OH D_3; D+100HD –2,500 I.U. vit. D_3 + 69 µg 25-OH D_3; D+150HD= 2,500 I.U. vit. D_3 + 103.5 µg 25-OH D_3; 25HD= 17.25 µg 25-OH D_3; 50HD= 34.5 µg 25-OH D_3; 100HD= 69 µg 25-OH D_3; 150HD= 103.5 µg 25-OH D_3.

Table 5-5. Effect of diet on Newcastle disease virus antibody titers[1]

Diet[2]	n	NDV 1° Antibody Response[3]	NDV 2° Antibody Response[4]	Δ NDV Antibody Response
D	8	5	2588	2583
D+25HD	8	7	1526	1518
D+50HD	8	8	2532	2523
D+100HD	8	4	2563	2559
D+150HD	8	2	1463	1460
25HD	8	6	1448	1441
50HD	8	1	2146	2144
100HD	8	3	2129	2126
150HD	8	8	2024	2015
	SEM	3.2	477.2	477.6
ANOVA			**Probabilities**	
Diet		0.7281	0.3936	0.3950

[1] At 28 and 35 days of age, 2 birds per pen were selected at random and vaccinated with a 4-way commercial vaccine for infectious bursal disease virus, Newcastle disease, infectious bronchitis and avian reovirus. Blood samples were obtained at days 35 and 41 to assess primary and secondary antibody responses. Only Newcastle disease virus antibody titers are reported.

[2]The following are the dietary treatments: D= 2,500 I.U. vit. D_3; D+25HD= 2,500 I.U. vit. D_3 + 17.25 µg 25-OH D_3; D+50HD=2,500 I.U. vit. D_3 + 34.5 µg 25-OH D_3; D+100HD –2,500 I.U. vit. D_3 + 69 µg 25-OH D_3; D+150HD= 2,500 I.U. vit. D_3 + 103.5 µg 25-OH D_3; 25HD= 17.25 µg 25-OH D_3; 50HD= 34.5 µg 25-OH D_3; 100HD= 69 µg 25-OH D_3; 150HD= 103.5 µg 25-OH D_3.

[3]Antibody response at 35 d of age, after first vaccination at 28 d of age.

[4]Antibody response at 41 d of age after second vaccination at 35 d of age.

CHAPTER 6: The Effect of Maternal Dietary Vitamin D Source on Fertility, Hatchability, Chick Quality and Progeny Growth and Bone Mineral Density

6.1. INTRODUCTION

Vitamin D_3 status of the broiler breeder can significantly affect the development of the chick embryo and subsequently its hatchability (Wilson, 1997). Previous work has shown that the vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 contents within the egg yolk (Mattila, et al., 1999). The egg yolk, which is the main source of nutrition for the developing embryo and newly hatched chick (Speake, et al., 1998), has a specific binding protein for vitamin D (Fraser and Emtage, 1976; White, 1987). This vitamin D binding protein has a high affinity for vitamin D_3 but also binds 25-OH D_3, and both are incorporated into the yolk (Edelstein, et al., 1973; Fraser and Emtage, 1976). When there is a high concentration of 25-OH D_3 in the blood of the hen, it can displace vitamin D_3 from the vitamin D_3 binding protein such that more 25-OH D_3 than otherwise gets passed into the yolk (Fraser and Emtage, 1976). Interestingly, the active vitamin D metabolite, $1,25(OH)_2D_3$ does not get passed from the hen into the egg yolk. Therefore if only $1,25(OH)_2D_3$ is provided to the hen, normal embryonic growth does not occur due to a vitamin D deficiency in the egg (Henry and Norman, 1978; Sunde, et al., 1978; Soares, et al., 1979; Ameenuddin, et al., 1983; Hart, et al., 1986; Ameenuddin, et al., 1987).

The metabolism of vitamin D_3 is important within the developing embryo. The enzyme, 25-hydroxyvitamin D-1α-hydroxylase, which is responsible for the hydroxylation of 25-OH D_3 to 1, $25(OH)_2D_3$ is present as early as 12 d of incubation and

increases in specific activity during further embryonic development (Turner, et al., 1987). Previous studies have shown that vitamin D_3 is hydroxylated to 25-OH D_3, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ within the chick embryos (Moriuchi and Deluca, 1974; Bishop and Norman, 1975; Kubota, et al., 1981). The early development of vitamin D metabolism within the chick signifies the importance of this nutrient to the developing embryo. Quail eggs that were deficient in vitamin D did not hatch because Ca transport across the chorioallantoic membrane to the embryo is dependent on 1,25$(OH)_2$ D_3 (Elaroussi and DeLuca, 1994; Elaroussi, et al., 1994). Therefore, vitamin D-deficient eggs would have limited formation of 1,25$(OH)_2$ D_3 to support the Ca transport from the eggshell to the embryo. However, even with diets sufficient in vitamin D (2,200 IU), the addition of 1,100 IU of 25-OH D_3 to the diet of turkey breeders improved egg hatchability as compared to dietary vitamin D_3 alone (Manley, et al., 1978). This may be related to the fact that 25-OH D_3 is more efficiently utilized by the bird (Bar, et al., 1980) which may also be the case for the chick embryo as well. In the broiler, 25-OH D_3 has been shown to increase BW and feed conversion efficiency (Yarger, et al., 1995). Therefore, improving the 25-OH D_3 status of the chick at hatch may improve the growth and production prospects of the chicks.

Providing the broiler breeder hen with a more available source of vitamin D activity for metabolism to 1, 25$(OH)_2$ D_3, such as 25-OH D_3, may increase the absorption efficiency of Ca from the gut, allowing more to be available for eggshell formation. In addition, maternal supplementation of 25-OH D_3, allows for more precursor to be available during egg formation and may result in increased egg hatchability and improved chick quality which could in turn result in better growth and feed conversion

of the broiler chick. Therefore the objective of the current research was to investigate the potential benefits of maternal dietary 25-OH D_3 on fertility, hatchability, chick quality, broiler production traits and plasma 25-OH D_3, and bone quality. We hypothesized that supplementation of the maternal diet with 25-OH D_3 would improve egg hatchability, chick quality as well as enhance progeny production performance, plasma 25-OH D_3 and bone quality.

6.2. MATERIALS AND METHODS

6.2.1. *Experimental Design and Conditions*

This experimental protocol was approved by the University of Alberta's Faculty Animal Policy and Welfare Committee in accordance with the CCAC guidelines (Canadian Council on Animal Care, 1993). Cobb 500 broiler hatching eggs (n=3,200) were obtained from a commercial breeder flock at 29 wk of age. Sister flocks of broiler breeders in 2 separate barns on the same production complex in the USA, that were of the exact same age and strain (Cobb 500) were used. Both broiler breeder flocks were housed at the same time. In 1 barn, in which half of the eggs (n=1,600) came from, the broiler breeders received 3,000 IU/kg of dietary vitamin D_3 (Control treatment) during the breeder phase. In the other barn in which the other half of the eggs (n=1,600) came from the breeder hens that were fed the same diet as the Control birds, but supplemented with 34.5 μg of 25-OH D_3 per L of water starting 3 weeks (26 wk of age) prior to fertile egg collection (25-OH D_3 treatment).

6.2.2. Hatching Eggs

Eggs were incubated (Jamesway single-stage incubator, Jamesway Incubator Company Inc, Cambridge, ON, Canada) for 21.5 d (temperature = 37.5 C and relative humidity = 85%). At 7 d of incubation, eggs were candled and any non-fertile or non-viable embryos were removed. Eggs were transferred to a hatcher (Jamesway single-stage hatcher, Jamesway Incubator Company Inc, Cambridge, ON, Canada) at 18 d of age, and placed in hatch baskets which held 18 eggs per basket. At hatch, stage of development at embryonic mortality (Early = 0 to 7 d; Late = 8 to 18 d), hatchability and chick BW were assessed for each maternal dietary treatment group.

6.2.3. *Egg Quality*

A subset of eggs (N=200 per treatment) were assessed for egg quality traits. Egg specific gravity was measured by the floatation method (Hamilton, 1982) with a series of saline solutions of increasing specific gravity ranging from 1.060 to 1.010 in increments of 0.002. Individual eggs were weighed, carefully broken and the yolk and albumen removed and separated; the individual weights of yolk, albumen and shell were recorded. Eggshell weight (with membranes attached) was measured after the eggshells were washed and air-dried overnight, after which eggshell thickness was determined on the egg shell from the middle of the egg using a micrometer. Eggshell conductance was determined using methods described by O'Dea et al., (2004) and calculations given by Ar et al., (1974). Briefly, the rate of egg weight loss (presumed to be moisture loss) was determined daily on eggs (n=15 per treatment) that were placed in desiccators and covered in desiccant for a 9 day period. Room temperature was recorded daily for the determination of the saturation vapor pressure.

6.2.4. *Broiler Production Trial*

Chicks from each maternal treatment group were randomly allocated to 20 floor pens at a rate of 50 birds per pen (14.09 birds/m^2), maintained in the maternal dietary treatment groups (n= 1,000 per maternal dietary treatment). Chicks were reared in a light-tight barn and given 1 h of light per day through incandescent lights. The birds were fed a crumbled starter ration (23 % CP, 3,067 kcal/kg ME, 1.1 % Ca, 0.77% available P and 2,500 IU of supplemental vitamin D_3) from 0 to 14 d of age. The grower (20 % CP, 3,152 kcal/kg ME, 0.90% Ca, 0.70% available P and 2,500 IU of supplemental vitamin D; fed from 15 to 27 d of age) and finisher (19 % CP, 3,196 kcal/kg ME, 0.85% Ca, 0.68% available P and 2,500 IU of supplemental vitamin D; fed from 28 to 41 d of age) rations were pelleted. All diets were wheat-based, supplemented with a commercial arabinoxylanase enzyme (Avizyme 1302, Danisco Animal Nutrition, Marlborough, UK) and formulated to meet or exceeded NRC and primary breeder nutrient recommendations. Pen BW of broilers were measured at 0, 7, 14, 27, and 42 d. Feed consumption and feed conversion ratio were assessed on a pen basis at 7, 14, 27, and 42 d of age. Individual BW were obtained at 0, 14 and 42 d to determine the coefficient of variation within the pen.

6.2.5. *Chick Plasma 25-OH D_3 Analysis*

During the first 2 weeks post-hatch, 10 chicks per maternal treatment were assessed for blood 25-OH D_3 levels every 2 days. Plasma 25-OH D_3 was measured using the HPLC method as outlined in Chapter 4.

6.2.6. *Bone Mineral Density Analysis*

Bone mineral density at 41 d was measured on the right femur of male birds (n=25 per treatment) selected at random from each maternal treatment group using the quantitative computed tomography procedure as per outlined in Chapter 2.

6.2.7. *Statistical Analysis*

The egg was the experimental unit for the egg trait data. Each hatch basket of 18 eggs was the experimental unit for the hatch data. The pen was the experimental unit for the broiler growth data. One pen from the maternal 25-OH D_3 treatment was removed from the data set due to flooding within the pen, which affected broiler production traits. Most data, except for the percentage data (% fertility, % hatch (total eggs), % hatch (fertile eggs), % early and late mortality, % late hatch, all the % internal and external pips (live and dead) as well as the % dead and % culls at hatch) were analyzed as a 1-way analysis of variance with maternal dietary treatment as the main effect, using the Mixed procedure in SAS (SAS Institute, 1999). The percentage data listed above, were analyzed as a chi-square analysis using SAS (SAS Institute, 1999). Effects over time were analyzed using the repeated measures procedure in Proc Mixed of SAS (SAS Institute, 1999). Means were compared using LSmeans comparisons of SAS (SAS Institute, 1999). Significance was assessed at a $P < 0.05$.

6.3. RESULTS AND DISCUSSION

6.3.1. *Fertility, Hatchability and Chick Quality*

There was a trend towards an improvement in hatchability of total and fertile eggs (P= 0.07; Table 6-1) as a result of a nearly 30% reduction in early embryonic mortality when broiler breeders were supplemented with 25-OH D_3 (P <0.03; Table 6-1). It has previously been shown that vitamin D-deficient quail embryos will expire in the later stages of embryonic development due to severe Ca deficiency (Elaroussi, et al., 1993). In the current study, this was not the case as there were no differences in late embryonic mortality (P=0.84; Table 6-1). However, neither treatment group, in the current study was deficient in vitamin D. In contrast to the current study, Antencio et al (2005b) found that a maternal diet supplementation of 3,125 ng 25-OH D_3 per kg of feed reduced late but not early embryonic mortality as compared to 3,125 ng vitamin D_3 per kg of feed. It should be noted that 3,125 ng/kg of 25-OH D_3 would provide a greater amount of vitamin D activity than 3,125 ng/kg of vitamin D_3 (Standing Committee on the Scientific Evaluation of Dietary Reference Intake, et al., 1997) and this explains the different results found in the current study. When the breeder dietary levels of 25-OH D_3 and vitamin D were increased to 12,500 ng/kg there was no difference in embryonic mortality (Atencio, et al., 2005b). Manley et al. (1978) found the addition of 1,100 ICU of 25-OH D_3 to a diet that already contained 2,200 ICU of vitamin D_3 to increase percent hatch of fertile turkey eggs from 48 wk old hens, with a % hatchability of 36.9 to 43.6 over 4 weeks compared to 12.5 to 28.2% for the eggs from the turkeys on the Control, non-25-OH D_3 supplemented treatment. That study did not report at what stage of embryo development the losses occurred so a direct comparison of time of embryonic death with the present study is not possible. In addition, an explanation of the overall low hatchability of eggs from the turkeys in this study was not given.

The hatch weight of chicks from the Control eggs was greater than those from the 25-OH D_3 eggs ($P<0.001$; Table 6-2). This was a result of the greater set and transfer weight of the eggs from the Control treatment ($P<0.0001$; Table 6-2). Egg size is 1 of the main factors affecting chick weight at hatch (Wilson, 1991). However, the eggs from the Control group also lost a greater amount of weight (~0.5 g difference) throughout the 21.5 d of incubation ($P<0.05$; Table 6-2). This may be explained in part by the greater percentage of eggshell of the eggs from the breeders the received the 25-OH D_3 (Table 6-3). It has previous been shown that water vapor conductance increases with egg weight (Ar, et al., 1974). In the current study, there was no treatment effect on eggshell conductance; however eggs were selected to be within 56.3 +/- 0.5 g egg weight thereby eliminating the effect of egg size (Table 6-2). However, eggs that were set for hatch were not of equal sizes among treatment groups, therefore it would be expected that the larger egg size would have a greater moisture loss during incubation.

6.3.2. *Egg Quality*

Egg weight of the samples of eggs used for egg quality was found not to be different among the 2 treatment groups. This is in contrast to the difference in egg weight shown in Table 6-2 for the eggs that were set for hatch. It is most likely the difference in replications that lead to the different egg weight results, with 200 eggs being selected for egg quality while over 1500 were set for hatch. A power calculation showed that an n=800 per treatment would be needed to find a significant difference in egg weight between the two treatments.

The addition of 25-OH D_3 to the water of the breeders resulted in an approximately 1.7% higher percentage of eggshell over eggs from those breeders on the Control treatment (Table 6-3). There was no difference in egg specific gravity between the 2 treatment groups (Table 6-3). As egg specific gravity is related to shell weight and thickness, there were also no effects of vitamin D treatment on these eggs traits as well (Table 6-3). This is contrary to a previous study that found a strong positive correlation between specific gravity and percent eggshell (Holder and Bradford, 1979). In the current study, although the % shell was different between the treatments, the difference may have been small enough that there was no measurable effect on specific gravity. However, similar to the results of the current study, supplementation of laying hens diets over a 16 to 20 week period with 25-OH D_3 in place of vitamin D did not improve egg specific gravity (Keshavarz, 2003). In addition, turkey egg specific gravity was not altered by the dietary supplementation of 25-OH D_3 in addition to vitamin D in comparison with dietary vitamin D_3 alone (Manley, et al., 1978).

The yolk of the eggs from the breeders that received the 25-OH D_3 represented a greater proportion of the egg than those from breeders that received only the dietary vitamin D_3 ($P<0.05$; Table 6-3). An increase in egg yolk caused by dietary 25-OH D_3 has not been reported in the literature. There was no significant difference between dietary treatments on % albumen (Table 6-3).

6.3.3. *Broiler Growth and Feed Efficiency*

The greater chick BW at hatch of the Control group (Table 6-2) did not have a significant effect on BW, broiler production characteristics or flock uniformity after that

time (Tables 6-4 and 6-5). There was a difference in chick BW at hatch between treatment groups at hatch (in the sections of the hatch basket; Table 6-2), and when chicks were randomly placed in floor pens (Table 6-4). The only maternal treatment effect on broiler production was found during the grower phase (15 to 27 d) in which the birds from the 25-OH D_3 maternal treatment had a lower feed conversion ratio (Table 6-4). Although not significant, BW and gain during the grower phase were greater for the broilers from the maternal 25-OH D_3 treatment (P=0.0592 and 0.0762 for BW and gain, respectively; Table 6-4), while there was no difference in feed consumption (P = 0.8320; data not shown), which resulted in a lower feed conversion of this treatment group versus the Control group (Table 6-4). Similarly, in previous work preformed in our lab (see Chapter 3), 25-OH D_3 fed to broilers resulted in differences in BW, BW gain and feed consumption in the grower phase (11 to 28 d) but not the starter phase (0-10 d; Table 4-2). However, unlike the current study, BW gain and not FCE were affected by dietary 25-OH D_3 in that experiment. By feeding graded levels of vitamin D_3 to broiler breeders, Atencio et al. (2005a) found BW of progeny to increase with increasing levels of maternal dietary vitamin D_3. Therefore supplementation of 34.5µg 25-OH D_3 per L in the water in addition to 3,000 IU of dietary vitamin D_3 to breeders did not affect BW gain of the progeny chicks, but reduced feed conversion ratio during the grower phase relative to the Control group.

6.3.4. *Broiler Chick Plasma 25-OH D_3 from hatch to 14 d*

Chick plasma 25-OH D_3 at hatch was not affected by maternal dietary treatment except at 4 d of age, when, levels were greater for the 25-OH D_3 maternal treatment than

the Vitamin D_3 treatment (Figure 6-1). The cause of the delay in the appearance of treatment differences in plasma 25-OH D_3 is unknown at this time, however we speculate it could be an effect of the rate of yolk sac resorption and the availability of these nutrients found within the yolk sac. Previous work has shown the yolk sac to be intensively absorbed during the first 5 d post-hatch (Jamroz, et al., 2004). This indicates that stored 25-OH D_3 (either tissue or yolk sac) may be readily available to the fast growing chick and may indicate a delay in the maturation of the mechanisms for liver 25-OH D_3 production from dietary vitamin D_3. Supplementation of 25-OH D_3 in the breeder diet resulted in increased plasma levels of this compound in the chicks only at 4 d post-hatch, indicating a transient effect of maternal supplementation (Figure 6-1). This may have been a result of the absorption of the metabolite from the yolk sac in the days following hatch.

In both treatment groups, plasma 25-OH D_3 decreased after hatch until 6 d of age, where it remained constant to 10 d, after which chicks from both maternal treatment demonstrated increasing plasma 25-OH D_3 levels. The results of the current study indicate that chicks appear to have similar levels of plasma 25-OH D_3, regardless of maternal supplementation. To the authors knowledge, there are no reports on the effect of maternal dietary 25-OH D_3 on the chick plasma 25-OH D_3. It has been shown that increasing dietary vitamin D_3 increases both egg yolk vitamin D_3 and 25-OH D_3 (Mattila, et al., 1999), however the effect of yolk vitamin D_3 and its metabolites on the plasma level of the hatching chick has not been previously reported. These results seem to indicate an impairment of conversion of dietary vitamin D_3 to 25-OH D_3 between 0 and 6 d of age. Our lab has previously shown 25-OH D_3 levels decrease after hatch if the

chicks are not provided dietary 25-OH D_3 (see Chapter 4; Figure 4-1). In support of this, previous research by Stevens et al (1984), found that kidney 1α-hydroxylase activity, which was greatest when vitamin D_3 was deficient, peaked at 8-12 d post-hatch in the progeny of poults from turkey hens consuming 2,700 IU of vitamin D_3. This shows that chicks from hens consuming adequate vitamin D_3 become most deficient in vitamin D_3 8 days after hatch (perhaps when yolk stores are used up). In rats, vitamin D_3 has been shown to be stored mainly in adipose tissue but has also been found to be stored in the kidney and liver (Rosenstreich, et al., 1971). The results of the current study may indicate that 25-OH D_3 is stored rather than circulating in the chicks from broiler breeders fed 25-OH D_3 which than may explain the improvement in production efficiency in the grower phase of the broiler production trial.

6.3.5. *Broiler Bone Mineral Density*

Femur total, cortical and trabecular BMD and cross-sectional areas of broilers at 41 d of age were not different between the 2 maternal dietary treatment groups (Table 6-6). This is not surprising as the plasma 25-OH D_3 levels only showed a significant maternal treatment effect at 4 d post-hatch (Figure 6-1). During this time bones are not well mineralized; maximum bone density is not reached until at 35 wk post-hatch (Rath, et al., 2000). In the current study, plasma 25-OH D_3 levels between the Control and 25-OH D_3 maternal treatment groups were equivalent from 0 to 2 and from 6 to 14 d post-hatch (Figure 6-1). Rapid bone growth and formation occurs up to 28 d of age in broilers (Leslie, et al., 2006). Therefore, it is likely that maternal dietary supplementation of 25-OH D_3 would not have long-term effects on broiler bone formation. Previous work

investigating the effect of increasing maternal dietary levels of vitamin D_3 (from 0 to 2000 IU as well as from 125 IU to 4000 IU of vitamin D_3 (Atencio, et al., 2005a) and from 300 to 2700 IU vitamin D_3; (Stevens, et al., 1984)) on bone ash of the broilers found that the greater the level of maternal dietary vitamin D_3, the greater the bone ash and reduction in tibial dyschrondroplasia (TD) score of the progeny (Stevens, et al., 1984; Atencio, et al., 2005a). Although bone ash was not measured in the current study, the significant positive correlation between BMD and bone ash observed in Chapter 3 would suggest that the 2 maternal treatments would have had equivalent bone ash contents. Therefore, maternal dietary 25-OH D_3 did not affect broiler bone mineral density at 41 d of age.

In conclusion, the main effect of maternal dietary 25-OH D_3 was the reduction in early embryonic mortality, resulting in a marginally greater hatch of fertile eggs; thereby indicating that 25-OH D_3 has some protective effect on the developing embryo from 0 to 10 d of age. This reduction in early embryonic mortality could result in a significant increase in return to hatching egg producers. However, minimal effects of maternal dietary vitamin D source on broiler growth and production efficiency were observed.

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Table 6-1. Effect of maternal vitamin D source on hatchability and fertility from 29 wk-old broiler breeders[1]

Treatment		% Fertility	% Hatch (total eggs)	% Hatch (Fertile Eggs)	% Early Mortality	% Late Mortality	% Late Hatch	% IPL[2]	% IPD[1]	% EPL[1]	% EPD[1]	% Dead	% Cull
Control[3]		95.70	84.66	88.46	6.22[a]	2.14	0.07	0.07	0.55	0.21	0.21	0.07	1.11
25-OHD$_3$[4]		96.05	86.91	90.48	4.37[b]	2.25	0.13	0.19	0.58	0.45	0.13	0.06	0.58
Chi Square	DF	Probabilities											
Treatment	1	0.6266	0.0717	0.0711	0.0242	0.8396	0.6118	0.3736	0.9247	0.2604	0.6006	0.9594	0.1187

[a,b]Treatment means within same column with different superscripts are significantly different (P<0.05).

[1]n for % Fertility and % Hatch (total eggs) was 1512 for the Control and 1619 for the 25-OH D_3; For the rest of the variables n for the Control = 1447 and 1555 for the 25-OH D_3.

[2]IPL = Internal Pip Live (chick pips through only membrane and is alive); IPD=Internal Pip dead (chick pips through only membrane and is dead); EPL= External Pip Live (chick pips through shell and is alive); EPD= External Pip Dead (chick pips through shell and is dead).

[3]Broiler Breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity.

[4]Broiler Breeders supplemented with 34.5µg of 25-OH D_3 per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D_3.

Table 6-2. Effect of maternal vitamin D source on set, transfer and weight loss of hatching eggs during incubation and chick hatch BW from 29 wk-old broiler breeders

Treatment	Set Egg Weight[1] (g)	Transfer Egg Weight[1] (g)	% Weight Loss[1]	Eggshell Conductance[1] (mg H_2O/d/mm Hg)	Chick BW (g)
Control[1]	56.0[a] (1510)[2]	49.9[a] (1399)	10.98[a] (1399)	11.32 (15)	38.2[a] (84)
25-OHD_3[2]	55.5[b] (1619)	49.6[b] (1523)	10.82[b] (1523)	11.76 (15)	37.7[b] (88)
SEM	0.09	0.09	0.05	0.42	0.10
ANOVA			Probabilities		
Treatment	<0.0001	0.0059	0.0088	0.4624	0.0004

[a,b] Treatment means within same column with different superscripts are significantly different ($P<0.05$). Set egg weight = weight of egg when first put in incubator; transfer egg weight = weight of egg after 18 d of incubation; % weight loss = % of weight loss of the egg from 0 to 18 d of incubation; eggshell conductance = rate of water loss from egg when stored for 7 d covered with desiccant.

[1] Broiler breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity.

[2] Broiler breeders supplemented with 34.5µg of 25-OH D_3 per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D_3.

[3] Means and followed by n in parenthesis; n for egg data = number of eggs for each treatment, n for BW = # of hatch basket sections (18 eggs/basket).

Table 6-3. Effect of maternal vitamin D source on egg quality from 29 wk-old broiler breeders

Treatment	n	Egg Wt (g)	Specific Gravity[1]	Shell Wt[1] (g)	Shell Thickness[1] (mm)	% Shell[1]	% Yolk[1]	% Albumen[1]
Control[2]	200	56.08	1.079	5.43	0.415	9.68[b]	29.44[b]	57.21
25-OHD$_3$[3]	200	55.66	1.080	5.47	0.414	9.84[a]	29.91[a]	56.87
SEM		0.255	0.0004	0.038	0.002	0.055	0.160	0.18
ANOVA	DF				Probabilities			
Treatment	1	0.2416	0.7195	0.4035	0.7295	0.0394	0.0421	0.1164

[a,b]Treatment means within same column with different superscripts are significantly different (P<0.05).

[1]Specific gravity was measured by the floatation method with a series of saline solutions of increasing specific gravity ranging from 1.060 to 1.010 in increments of 0.002. Shell wt is the weight of the washed and air-dried egg shell (with membrane). Shell thickness was determined on the egg shell from the middle of the egg using a micrometer. Percent shell, yolk and albumen were determined as a percentage of the total egg weight.

[2]Broiler breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity.

[3]Broiler breeders supplemented with 34.5µg of 25-OH D_3 per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D.

Table 6-4. Effect of maternal vitamin D source on broiler BW, gain and FCR from 0 to 41 d

		BW (g)					Gain (g/bird/d)				
Treatment	n	Day 0	Day 7	Day 14	Day 27	Day 41	Day 0-7	Day 8-14	Day 15-27	Day 28-41	Overall
Control[1]	20	38.2[a]	138	367.7	1205	2338.4	14.2	28.4	70.3	82.4	55.0
25-OHD$_3$[2]	19	37.8[b]	139	370.1	1230	2362.4	14.3	28.8	71.9	82.7	55.7
SEM		0.14	1.4	2.50	9.0	14.46	0.19	0.24	0.64	0.88	0.36
ANOVA		Probabilities									
Treatment		0.0301	0.7026	0.4989	0.0592	0.2537	0.6667	0.2615	0.0762	0.8124	0.2062

		Feed Consumption (g/bird/d)					FCR				
Treatment	n	Day 0-7	Day 8-14	Day 15-27	Day 28-41	Overall	Day 0-7	Day 8-14	Day 15-27	Day 28-41	Overall
Control	20	16.3	52.8	100.0	160.4	92.9	1.15	1.85	1.42[a]	1.95	1.69
25-OHD$_3$	19	16.4	53.8	100.5	161.3	93.5	1.14	1.87	1.39[b]	1.95	1.68
SEM		0.15	0.47	0.85	1.16	053	0.01	0.02	0.01	0.02	0.01
ANOVA		Probabilities									
Treatment		0.8587	0.1275	0.6921	0.5828	0.4051	0.4209	0.3896	0.0250	0.8479	0.4337

[a,b]: Treatment means within same column with different superscripts are significantly different ($P<0.05$).

[1]Broilers from broiler breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity.

[2]Broilers from broiler breeders supplemented with 34.5µg of 25-OH D_3 per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D_3.

Table 6-5. Effect of maternal vitamin D source on BW coefficient of variance (CV) of broiler chicks

Treatment	n	Day 0 CV	Day 14 CV	Day 41 CV
Control[1]	20	7.5	11.5	11.78
25-OHD$_3$[2]	19	7.4	11.6	11.37
SE		0.20	0.33	0.40
ANOVA	DF		Probabilities	
Treatment	1	0.6747	0.9221	0.4774

[1]Broilers from broiler breeders fed a diet containing 3,000 IU of vitamin D$_3$ as the sole supplemental source of vitamin D activity.
[2]Broilers from broiler breeders supplemented with 34.5µg of 25-OH D$_3$ per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D$_3$.

Table 6-6. Effect of maternal vitamin D source on femur BMD and area of 41 d-old broilers

		Femur Density (mg/cm^3)			Femur Cross-Sectional Area (mm^2)		
		Cortical[1]	Trabecular[2]	Total[3]	Cortical	Trabecular	Total
Treatment	**n**						
Control[4]	25	908.25	83.24	475.33	31.37	34.31	67.49
25-OHD_3[5]	23	916.75	78.16	469.88	29.61	33.50	64.77
	SE	6.04	2.77	9.72	1.05	1.35	2.13
ANOVA				Probabilities			
Treatment		0.3154	0.2060	0.6998	0.2518	0.6778	0.3618

[1]Cortical = measurements taken on the area define as >500 mg/cm^3 and the outer part of the bone.
[2]Trabecular = measurements taken in the inner part of the bone in the trabecular space.
[3]Total = the total for the entire bone.
[4]Broilers from broiler breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity.
[5]Broilers from broiler breeders supplemented with 34.5µg of 25-OH D_3 per L of water starting 26 weeks of age in addition to 3,000 IU/kg of dietary vitamin D_3.

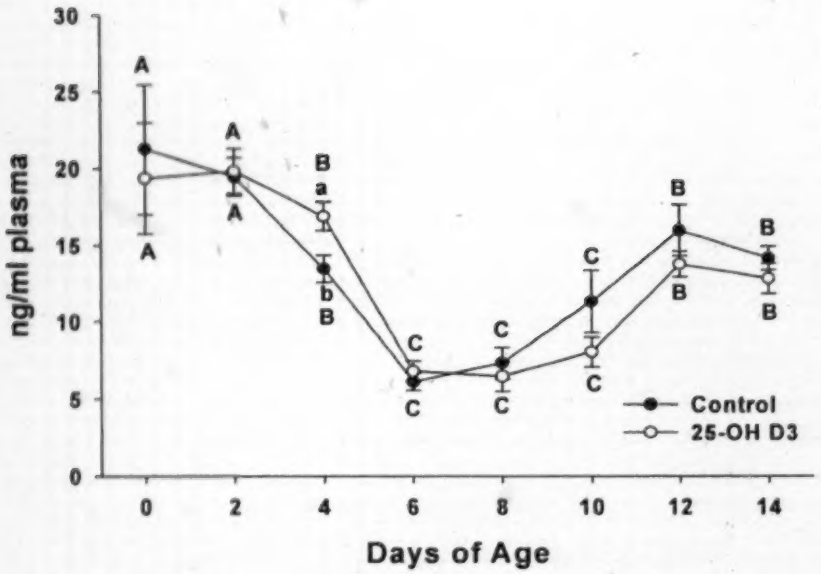

Figure 6-1. The effect of maternal vitamin D source on broiler chick plasma 25-OH D_3 from hatch to 14 d.
The control treatment was chicks from broiler breeders fed a diet containing 3,000 IU of vitamin D_3 as the sole supplemental source of vitamin D activity. The 25-OH D_3 treatment was chicks from broiler breeders supplemented with 34.5µg of 25-OH D_3 per L of water in addition to 3,000 IU/kg of dietary vitamin D_3. n = 12 birds per treatment at each sample day. $^{a-b}$Means labeled with lower case letters are significantly different at that age ($P<0.05$). $^{A-C}$Means across time within treatment with differing capital letters are significantly different over time ($P<0.05$).

CHAPTER 7: The Effect of Maternal Vitamin D Source on Broiler Breeder Production, Egg Quality, Hatchability and Bone Mineral Density, and Progeny *In Vitro* Early Innate Immune Function

7.1. INTRODUCTION

Twenty-five hydroxychocalciferol (25-OH D_3) is a metabolite of vitamin D_3 that is formed in the liver (Soares, et al., 1995). This metabolite is then hydroxylated to $1,25(OH)_2D_3$ in the kidney by 25-hydroxy-D_3-1α-hydroxylase (Norman and Hurwitz, 1993). $1,25(OH)_2D_3$ is considered the active form of vitamin D as it is this vitamin D metabolite that exerts its actions on calcium metabolism and cellular differentiation (Norman and Hurwitz, 1993).

Vitamin D is required for normal and successful embryonic development of poultry species. Previous work has shown that the vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 contents within the egg yolk (Mattila, et al., 1999). The egg yolk, which is the main source of nutrition for the developing embryo and newly hatched chick (Speake, et al., 1998), has a specific binding protein for vitamin D (Fraser and Emtage, 1976; White, 1987). When there is a high concentration of 25-OH D_3 in the blood of the hen, it can displace vitamin D_3 from the vitamin D_3 binding protein such that more 25-OH D_3 than otherwise gets passed into the yolk (Fraser and Emtage, 1976). Interestingly, the active vitamin D metabolite, $1,25(OH)_2D_3$ does not get passed from the hen into the egg yolk. Therefore if only $1,25(OH)_2D_3$ is provided to the hen, normal embryonic growth does not occur due to a vitamin D deficiency in the egg (Henry and Norman, 1978; Sunde, et al., 1978; Soares,

et al., 1979; Ameenuddin, et al., 1983; Hart, et al., 1986; Ameenuddin, et al., 1987). Quail eggs deficient in vitamin D did not hatch because Ca transport across the chorioallantoic membrane to the embryo is dependent on $1,25(OH)_2 D_3$ (Elaroussi and DeLuca, 1994; Elaroussi, et al., 1994). Therefore, vitamin D-deficient eggs would have limited formation of $1,25(OH)_2 D_3$ to support the Ca transport from the eggshell to the embryo. However, even with diets sufficient in vitamin D (2,200 IU), the addition of 1,100 IU of 25-OH D_3 to the diet of turkey breeders improved egg hatchability as compared to dietary vitamin D_3 alone (Manley, et al., 1978). This may be related to the fact that 25-OH D_3 is more efficiently utilized by the bird (Bar, et al., 1980) which may also be the case for the chick embryo as well. In the broiler, dietary 25-OH D_3 has been shown to increase BW and feed conversion efficiency (Yarger, et al., 1995). Therefore, increasing the 25-OH D_3 status of the chick at hatch may improve the growth and production potential of the chicks as there appears to be an impairment in the metabolism of vitamin D in newly hatched chicks when fed vitamin D_3 as previously reported in other studies of this thesis (see Chapters 4 and 6).

The innate immune system is the first line of cellular defense of the bird, the heterophils, monocytes and macrophages of this system work to recognize, phagocytise (engulf bacteria) and, using non-specific properties, kill the invading pathogen as well as working to signal the acquired immune response (Zekarias, et al., 2002). Some reports indicate that fast growth rates of commercial broilers and turkeys have had a negative impact on the immune response, making modern birds more susceptible to infections than in the past (Bayyari, et al., 1997; Yunis, et al., 2000). The group of chickens most at risk for infection and disease is the young, newly hatched chick (<1 wk of age), as

various aspects of the immune system are not functionally mature at this young age (Lowenthal, et al., 1994; Wells, et al., 1998).

Vitamin D is involved in various aspects of the immune system. Reports on the involvement of vitamin D in the immune function of poultry species are limited but have shown a depressed cellular immune response in vitamin D-deficient chicks (Aslam, et al., 1998). However no difference was reported between vitamin D or 25-OH D_3 fed chicks in 21 d macrophage nitric oxide production and cytotoxicity as well as 35 d cutaneous basophil hypersensitivity at adequate levels of each (Fritts, et al., 2004). In humans, one of the interesting roles of vitamin D in cellular differentiation is the promotion of maturation of monocytes into macrophages (Manolagas, et al., 1985; Provvedini, et al., 1986) and increasing the survival of these cells at the higher body temperatures that accompany an inflammatory immune response (Brown, et al., 1999). Furthermore, providing human and bovine cells *in vitro* with $1,25(OH)_2D_3$, induces macrophage nitric oxide production (Rockett, et al., 1998; Waters, et al., 2001). The other major mammalian phagocytic cells, neutrophils, have increased *in vitro* chemotactic activity in the presence of the vitamin D binding protein added to the media (Kew and Webster, 1988; Binder, et al., 1999).

The avian immune system begins to develops very early in ovo (Sharma, 1997). Therefore the maternal diet could have significant effects on the immunocompetence of the chick at hatch as it is the nutrients from the egg that will enable the chick to develop. The abundant number of studies indicating a regulatory role for vitamin D and its metabolites within the immune system of various other species suggests the possibility of similar roles within the poultry species, although little research has been done to

support this. Therefore the objectives to the current research were to investigate the effects of maternal dietary 25-OH D_3 supplementation on broiler breeder production traits and bone mineral density (BMD) as well as *in vitro* innate immune function of the progeny. It was hypothesized that maternal dietary 25-OH D_3 supplementation would support normal broiler breeder production, improve BMD as well as lead to a more mature innate immune system of their progeny at hatch.

7.2. MATERIALS AND METHODS

7.2.1. *Experimental Diets*

Wheat-based basal maternal diets devoid of supplemental vitamin D were formulated for each breeder phase to meet or exceed current primary broiler breeder (Aviagen™, Huntsville, AL, USA) and NRC (National Research Council, 1994) recommendations. Each basal diet was subdivided and supplemented with either 2,760 IU of dietary vitamin D_3 (Rovimix D3 500®, DSM Nutritional Products Inc., Parsippany, NJ) per kg of feed or 69 µg of dietary 25-OH D_3 (Rovimix HyD®, DSM Nutritional Products Inc., Parsippany, NJ) per kg of feed, which according to the manufacturer is the equivalent of 2,760 IU of vitamin D_3 activity, as the sole source of vitamin D activity. The hens were fed a broiler breeder Phase 1 ration (16.2% CP, 3.1% Ca, 0.43% aP and 2,870 Kcal/Kg ME) from 23 to 44 wk of age. From 45 to 65 wk of age hens were fed a Phase 2 ration containing 15.8% CP, 3.3% Ca, 0.37% aP and 2,870 Kcal/Kg ME.

7.2.2. *Experimental Design and Data Collection*

This experimental protocol was approved by the University of Alberta Faculty Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 1993). At 23 wk of age, 98 Ross 308 broiler breeder hens were randomly allocated to 4 floor pens (24 to 25 birds per pen; 2 pens per treatment; average BW of 2,273 ± 27) with straw covering the floor. Each pen provided a total of 8.3 m^2 of total floor space which included a raised wire slat area. Birds were raised in light tight barns. At 23 wk of age, light (through incandescent lighting) was increased from 10 h of light per day to 14 h of light per day, light was then increased again to 15 h per day at 25 wk and then 16 hr per day at 27 wk for the remainder of the trial. Birds were weighed and feed allocation adjusted on a weekly basis for the average BW of the 4 pens to maintain the breeder-recommended BW curve. Egg production was recorded on a daily basis for each pen. Fresh egg quality traits (egg weight, specific gravity, shell weight and thickness, yolk weight and albumen height and weight) were assessed every 6 weeks starting at 29 wk of age on all eggs from 2 consecutive days of egg production per period using the procedures outlined previously in Chapter 6. At 64 wk of age, blood from 10 birds per pen was obtained by brachial venipuncture, plasma was separated by centrifugation at 2000 rpm for 15 min and frozen at -20 C for subsequent analysis of 25-OH D_3 by HPLC (See chapter 4 for complete analytical procedure). At 65 wk of age, all broiler breeders were killed by cervical dislocation and body composition (weights of whole breast, *Pectoralis* major, *Pectoralis* minor, fat pad, liver and spleen) and ovarian morphology (weight of the ovary, oviduct, stroma and number of large, small and atretic yellow follicles) were assessed.

Initial (23 wk) right tarsometatarsus bone mineral density (BMD) was assessed on 12 live birds (6 per treatment group) prior to receiving any dietary treatment by quantitative computed tomography using a Stratec Norland XCT (XCT Research SA, Norland Corp., Fort Atkinson, Wisconsin, USA) scanner having a 50 kV x-ray tube as described by Riczu et al (2004). BMD was then assessed on 8 live birds per pen at 30 and 49 wk of age and on excised bones of those same birds at 65 wk of age.

Hens were artificially inseminated at 3 ages; from 31 to 33 wk of age, from 46 to 48 wk of age and from 61 to 63 wk of age. At each age, broiler breeders were inseminated 3 times over a 2 week period (2 days in a row at the beginning and once 7 d later) with 50 µl of pooled semen from approximately 15 Ross 344 roosters. Eggs from each of these time periods were collected 2 days after hens were first inseminated, incubated and hatched. At each breeder age, eggs were collected for 7 d and incubated as a single group; eggs were then collected for 7 additional d before being incubated, thus resulting in 2 complement hatches for each broiler breeder age. Eggs were incubated (Jamesway single-stage incubator, Jamesway Incubator Company Inc, Cambridge, ON, Canada) for 21.5 d (temperature = 37.5 C and relative humidity = 85%). At 7 d of incubation, eggs were candled and any non-fertile or non-viable embryos were removed. Eggs were transferred to a hatcher (Jamesway single-stage hatcher, Jamesway Incubator Company Inc, Cambridge, ON, Canada) at 18 d of incubation, and placed in hatch trays divided into 8 sections, which held 18 eggs per section. At hatch, embryonic mortality, hatchability and chick BW were assessed for each maternal dietary treatment group. Percent fertility was calculated as the percentage of fertile eggs out of the total number of eggs set for incubation. Percent hatch of total was calculated as the percentage of chicks

that hatched from the total number of eggs set for incubation. Percent hatch of fertile eggs was calculated as the percentage of chicks that hatched from only fertile eggs. In addition, early (0 to 7 d) and late (8 to 21 d) embryonic mortality, late hatch (chicks requiring longer than 21.5 d to hatch), % internal pip live and dead (IPL, and IPD; those chicks that piped through the shell membrane only prior to expiration), % external pip live and dead (EPL and EPD; those chicks that piped through the shell prior to expiration) were determined as a percentage of fertile eggs, which were determined through breakout of non-hatched egg.

Eggshell conductance was determined on a subset of eggs (15 per maternal treatment) collected the day after the final eggs were collected for hatching at each broiler breeder age. This procedure was performed using methods described by (O'Dea, et al., 2004) and calculations given by (Ar, et al., 1974). Briefly, rate of egg weight loss was determined daily on eggs that were placed in desiccators and covered in desiccant for a 9 day period. Room temperature was recorded daily for the determination of the saturation vapor pressure.

After hatch, chicks were separated based on maternal vitamin D treatment and housed in Petersime battery brooders (Petersime Incubator Co., Gettysburg, OH) for 1 wk post-hatch and fed a standard broiler mash ration (23.25% CP, 1.1% Ca, 0.55% aP, 3,134 Kcal/kg ME and 2,500 IU vitamin D_3) devoid of any antibiotic supplementation. At 1 and 4 d post-hatch, blood was collected from 15 female chicks per maternal treatment per day and plasma 25-OH D_3 was determined by HPLC (see Chapter 4 for HPLC procedure). Previous research has found no difference in plasma 25-OH D_3 between males or females consuming the same diet (see Chapter 4). At 1 and 4 d post-hatch, male

chicks (n=15 chicks per maternal treatment) were assessed for *in vitro* innate immune function. Approximately 1 ml of whole blood was collected into heparinized 5 ml vacutainer tubes through decapitation. Under sterile conditions, 40 µl of whole blood was removed for each of the phagocytosis and bactericidal assays. The remainder of the whole blood was used to assess oxidative burst response. Therefore, all 3 *in vitro* immune assays were performed on blood from each of the 15 chicks per treatment. Both heparinized and ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was tested in each of the *ex-vivo* innate immune function assays, and heparinized anti-coagulated blood was found to yield more consistent results with the procedures used. In addition, EDTA interferes with Ca^{2+} ions involved in phagocytosis (van Eeden, et al., 1999).

7.2.3. *Phagocytosis Assay*

Peripheral blood cells were assessed for capability to phagocytize fluorescently-labeled *E. coli* as outlined in Millet et al. (2007) with some modification for analysis by flow cytometry. Forty µl of whole blood was diluted 1:20 with CO_2-independent media (Gibco, Invitrogen Corportion, Burlington, ON, CA) supplemented with 100 µg/ml of penicillin, 100 µg/ml of streptomycin and 4 mM L-Glutamine. Diluted blood and *E. coli* (K-12 strain; Molecular Probes (E-2864), Invitrogen Corportion, Burlington, ON, CA) were mixed together at a leukocyte to *E. coli* particle ratio of 1:100 and allowed to incubate for 15 minutes at 41 C. After submerging in an ice bath for 5 minutes to stop the phagocytosis reaction, each sample was then washed twice with 300 µl of CO_2-independent media supplemented with 100 µg/ml of penicillin and 100 µg/ml of

streptomycin to remove all non-phagocytized bacteria. The cell pellets were then lysed (ddH_2O + 0.83% w/v NH_4Cl + 0.17% w/v $NaHCO_3$ + 0.2% EDTA) to remove RBC from the mixture and fixed with methanol. Samples were then reconstituted in clear wash buffer (0.5 g bovine serum albumin (BSA), 1 ml EDTA and 500 ml Hanks balanced salt solution (HBSS)), and transferred to a sterile tube and stored on ice for subsequent measurement of fluorescence by flow cytometry (Becton-Dickinson FacScan Flowcytometer, Sunnyvale, CA). Cells were separated based on a slight modification of the method described by Holloway et al. (2003), using forward (cell size) and side scatter (granularity) characteristics. As it was difficult to distinguish between leukocyte subpopulations, for this assay the analysis included the entire white blood cell population after 10,000 events had been acquired. The percent of cells phagocytosing was assessed as the number of cells that had taken up at least 1 *E. coli* particle (ie. exhibiting increased fluorescence). Fluorescence of non-phagocytosing cells were determined from a control sample to which no fluorescent bacteria was added. The amount of bacteria taken up by each WBC was assessed using the mean fluorescence which was indicative of the amount of *E. coli* particles that were engulfed by an individual cell.

7.2.4. *Oxidative Burst Assay*

7.2.4.1. *Isolation of Peripheral White Blood Cells*

Whole blood samples were diluted in 1% BSA (1:2 v/v) and separated over a 1.119 density gradient (Sigma-Aldridch Canada Ltd. Oakville, ON) by centrifugation for 30 minutes. After centrifugation, the 1.119 band was collected and cells washed with 1%

BSA in PBS and centrifuged again. Cell pellets were then re-suspended to 1 ml of HBSS supplemented with BSA and EDTA.

Cell viability was determined by trypan blue exclusion assay as described by Holloway et al. (2003). Briefly, 50 µl of trypan blue stain (Sigma-Aldridch Canada Ltd. Oakville, ON) was added to 50 µl of diluted cell solution and viable (clear) and non-viable (blue) cells were counted with a hemocytometer. Cell viability was determined to be >90%.

7.2.4.2. *Assay Procedure and Analysis*

Oxidative burst was measured using a modified version of that given by He et al., (2003). 10 µl of 2', 7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldridch Canada Ltd. Oakville, ON) at a concentration of 10 µl/ml was added to re-suspended isolated granulocytes and incubated at 37 C for 5 minutes. After 5 minutes, 200 µl were removed and placed in a FACScan tube and stored on ice in the dark for subsequent analysis of cell background fluorescence by flow cytometry. 10 µl of PMA (Biomol, Plymouth Meeting, PA) at a concentration of 10 µg/ml were then added and 200 µl were removed at 5 min intervals for 20 min and placed in sterile tubes for subsequent analysis of fluorescence by flow cytometry. The oxidation of DCFH-DA produces a fluorescent product 2', 7'-dichlorofluorescein (DCF) in response to reactive oxygen species produced by phorbol myristate acetate (PMA)-stimulated heterophils (He, et al., 2003). Cells were separated based on forward (size) and side (granularity) scatter characteristics (Holloway, et al., 2003), which allowed for the separation of the leukocyte subpopulations. As heterophils are one of the major circulating innate immune cells in the bird (Maxwell and

Robertson, 1998), these cells were gated and used for the measurement of the oxidative burst response, after 20,000 events had been acquired. The extent of the oxidative burst response was measured as a ratio of increase in fluorescence from the non-stimulated background fluorescence of each sample.

7.2.5. *Bactericidal Assay*

White blood cells were assessed for bactericidal capability to *E. coli* as described by Millet et. al. (2007). Briefly, a stock solution of *E. coli* was prepared from the dilution of 1 *E. coli* pellet (MicroBiologics Inc., Saint Cloud, MN, USA) in 40 ml of sterile phosphate buffer saline (pH 7.4). A working solution was prepared by diluting 2 ml of the stock *E. coli* solution with 8 ml of PBS. Total bacterial counts of the working solution were obtained in duplicate each day the assay was performed to standardize the bacterial challenge to the cells. Forty µl of heparinized blood was diluted (1:10) with Gibco CO_2 independent media supplemented with 4 mM L-Glutamine. Forty µl of the working *E. coli* solution was then added to each sample of diluted blood resulting in approximately 250 *E. coli* particles per 50 µl of diluted blood. This mixture was incubated for 90 min at 41 C and then 50 µl of each sample was plated on Petri dishes with tryptic soy agar. Plates were incubated overnight at 37 C and counts of the total bacteria in 50 µl of the working solution and of the number of non-killed bacteria from the diluted blood and *E. coli* solutions were obtained. Percent killing was calculated as follows:

$$\frac{\text{Total of } \textit{E. coli} \text{ particles in working solution added} - \text{live } \textit{E. coli} \text{ colonies counted}}{\text{Total of } \textit{E. coli} \text{ particles in working solution added}} * 100$$

There is no killing data presented for 1 d post-hatch at the broiler breeder age of 31 to 33 wk as the bacteria were not available at the time of sample collection.

7.2.6. *Statistical Analysis*

The experimental unit of the broiler breeder data was the pen, for the hatch data was the egg, for the chick production data was the pen and for the chick immune function data was the individual chick. Breeder BW, egg production and quality, BMD and cross-sectional area, carcass characteristics and ovarian morphology, and progeny BW, growth and production efficiency, plasma 25-OH D_3, *ex vivo* phagocytosis and oxidative burst were analyzed was a 1-way analysis of variance with maternal dietary treatment as the main factor using the Mixed Model analysis in SAS (SAS Institute, 1999). The hatch data (% fertility, % hatch, % hatch of fertile eggs, % early and late mortality, % late hatch, % internal and external pips (live and dead), % dead and % culls) and % *E. coli* killed data was analyzed as a chi-square analysis using SAS (SAS Institute, 1999). Repeated measures analysis of SAS (SAS Institute, 1999) was performed on the plasma 25-OH D_3 and immune assay results to determine effects of broiler breeder age on those variables. Probability of differences was assessed at $P<0.05$. Means were separated using LSMeans comparisons (SAS Institute, 1999).

7.3. RESULTS AND DISCUSSION

7.3.1. *Effect of Dietary vitamin D Source on Broiler Breeder BW, Egg Production and Quality*

Dietary 25-OH D_3 did not significantly affect breeder hen BW (Figure 7-1). This was expected as broiler breeders were feed restricted to a specific target BW curve recommended by the Ross 308 breeder management guide to maximize egg production (Aviagen, 2002). In broilers, BW was increased in birds that consumed dietary 25-OH D_3 but only through increasing feed consumption (Chapter 4; Table 4-2), as broiler breeders are feed restricted to maintain a specific BW, increasing feed consumption was not possible for these birds. It is worth noting that birds used in this study were obtained at a lower average BW than the recommended target BW (Figure 7-1). Throughout the entire study, the broiler breeders were below the specific target BW but were fed (Fig 7-1) to maintain the target BW curve and egg production (Figures 7-2 and 7-3). All pens of broiler breeders received the same amount feed on a daily basis.

Dietary 25-OH D_3 did not affect breeder total settable egg production (Table 7-3). The breeder hens that received dietary 25-OH D_3 had a greater egg production at 28 wk than the control group (Figure 7-3). However, the 25-OH D_3 birds maintained the same egg production as the Control birds throughout the rest of the trial (Figure 7-3). There was a significant drop in egg production at 31 wk which was due environmental control issues and –40 C outside temperatures (Figure 7-3). Once resolved, egg production returned to target levels. Egg weight, egg specific gravity, % yolk, and % egg shell were not different between the 2 treatments at any age (Table 7-2). This could be due to the small replication number of pens (n= 2 per treatment). However, eggshell thickness at broiler breeder age of 29 wk was greater for the eggs from the broiler breeders on the 25-OH D_3 treatment (Table 7-2). These results, for the most part, are in agreement with the findings of Keshavarz (1996) and Soares et al. (1982), who found that 25-OH D_3 fed to

laying hens resulted in the similar egg production, egg weight and egg shell quality as those hens fed dietary vitamin D_3. Similarly, Keshavarz (2003), comparing the same level of vitamin D and 25-OH D_3 as used in the current study found no difference in egg specific gravity, shell weight, and % shell of eggs from laying hens. However, vitamin D is involved in Ca metabolism and therefore egg shell formation, and could be the reason egg shell thickness was greatest at 29 wk for the broiler breeders fed the 25-OH D_3. As this effect was not carried forward for the rest of the laying cycle, it therefore would not be expected to impact on broiler breeder production. At a broiler breeder age of 58 wk the % albumen was greatest for the eggs from birds on the Control diet. However, the albumen height was greatest for the eggs from hens on the 25-OH D_3 treatment at 52 wk (P=0.07) and 58 wk (P= 0.04; Table 7-2). A greater albumen height has been shown to decrease embryo weight and increase hatchability of the developing embryo in hatching eggs (Hurnik, et al., 1978; Deeming, 1989; Lapao, et al., 1999). In addition, as albumen height decreases with egg storage time (Lapao, et al., 1999), the eggs from the hens fed the 25-OH D_3 may be able to withstand longer egg storage time with less effect on embryo viability than those eggs from the control.

Previous research has demonstrated increased levels of plasma $1,25(OH)_2$ D_3 of laying hens during shell calcification (Abe, et al., 1979; Castillo, et al., 1979). This would lead to increased Ca absorption from the gut as well as bone Ca resorption (DeLuca, 2004) in response to the increased Ca demand for eggshell formation. In addition, it has been speculated that $1,25(OH)_2D_3$ is involved in the transport of Ca across the uterine membrane (Bar and Hurwitz, 1973), thereby, potentially increasing the amount of calcium available for eggshell deposition. In the current study, 25-OH D_3 had

no significant effect on egg shell quality over dietary vitamin D_3 except at 29 wk of age when shell thickness was greatest on eggs from breeders fed the 25-OH D_3 diet (Table 7-2). However, that was the only difference that reached significance over the entire production cycle of the broiler breeders. Keshavarz (1996, 2003) also found that the addition of dietary 25-OH D_3 did not affect egg shell quality of laying hens as compared to dietary vitamin D_3. McLoughlin and Soares (1976) also reported no effect of dietary 25-OH D_3 on egg shell quality of young hens, however, in older laying hens the addition of dietary 25-OH D_3 improved shell quality versus dietary vitamin D_3. The results of the current study also show that feeding 69 µg/kg of feed of 25-OH D_3 in place of vitamin D_3 supported production of eggs of the same quality. Previous research has found dietary 25-OH D_3 to produce eggs of similar quality as dietary vitamin D_3 whereas its more biologically potent metabolite, 1α-OH D_3, feed in excess of 6.8 µg/kg was found to be detrimental to the laying hen, reducing egg production and eggshell quality (Soares, et al., 1982).

7.3.2. *Effect of Dietary vitamin D Source on Broiler Breeder Bone Mineral Density and 65 wk Carcass Characteristics and Ovarian Morphology*

Live and excised right tarsometatarsus BMD were not different between the treatment groups from 32 to 65 wk (Table 7-3). Previous research has shown that dietary 25-OH D_3 is more efficiently absorbed from the gut than vitamin D_3 (Bar, et al., 1980). In the current study, it was hypothesized that this would be especially important in the broiler breeder because they are feed restricted and are unable to consume additional Ca in response to anticipated need for eggshell production as has been observed in the laying

hen (Roland, et al., 1973). However, unlike typical commercial laying hen facilities where hens are kept in cages, broiler breeders are normally housed on the floor and may be able to get sufficient exercise to maintain bone integrity, as structural bone loss is reduced when the bird is allowed more movement than what is available when housed in cages (Fleming, et al., 1994). Broiler breeders hens have greater body size and lower egg production, therefore calcium demand on the broiler breeder hen due to eggshell formation would be much less than for the laying hen.

Dietary vitamin D source did not affect body composition or ovarian morphology (Table 7-4). This suggests that dietary 25-OH D_3 fed at 69 µg/kg of feed supported equivalent growth of the broiler breeder hen as compared to 2,760 IU of vitamin D_3. In addition, at these vitamin D activity levels we would not expect to see any deficiency signs as both diets are well above the vitamin D requirement for poultry (National Research Council, 1994).

7.3.3. *Effect of Maternal Dietary Vitamin D Source on Fertility, Hatchability, Chick BW and Early Chick Performance*

At 31 to 33 wk of breeder age, there was a greater % fertility of eggs from broiler breeders that received the vitamin D_3 diet, 91.88 vs 85.62 ($P = 0.01$) for the vitamin D_3 and 25-OH D_3 treatments, respectively (Table 7-5). However, there was no difference between the 2 maternal dietary treatments in % hatch of fertile eggs (76.28 vs 76.97; $P = 0.99$) for the vitamin D_3 and 25-OH D_3 treatments, respectively). This was due to the greater % of total embryonic mortality of the Control treatment at 31 to 33 wk (27.41 vs 18.43; $P = 0.002$), for the Control and 25-OH D_3 treatments, respectively; Table 7-5).

There were no treatment effect on IPL, IPD, EPL, EPD, culls and dead chicks at hatch at 31 to 33 wk (Table 7-5). Although there were no differences in set or transfer egg weight and eggshell conductance, chick BW at hatch was significantly greater for the chicks from broiler breeders fed 25-OH D_3 at 31 to 33 wk (37.54 vs 38.17; P = 0.02; SEM = 0.20), for the Control and 25-OH D_3, respectively; Table 7-6).

At 46 to 48 wk broiler breeder age, there were no treatment effects on any of the hatch variables (fertility, hatchability, embryonic mortality and late hatch) or chick BW (Table 7-5 and 7-6).

At 61 to 63 wk of age, there was a difference in % hatch of total eggs and % hatch of fertile eggs (78.38 vs 84.62 and 86.23 vs 91.07 for the Control and 25-OH D_3 treatments, respectively; Table 7-5). However, there were no significant differences in embryo mortality, late hatches, internal and external pips as well as culls and dead chicks at hatch at 61 to 63 wk (Table 7-5). In addition there were no treatment effects on set and transfer egg weight, % egg weight loss from set to transfer, eggshell conductance as well as chick BW at hatch at 61 to 63 wk broiler breeder age (Table 7-6).

In contrast to the current study, Atencio et al (2005) found that a maternal supplementation of 3.125 µg/kg 25-OH D_3 reduced late embryonic mortality as compared to 3.125 µg/kg of vitamin D_3. Twenty-five-OH D_3 is much more potent than vitamin D_3. To the authors' knowledge, there have been no reported values for the relative activity of 25-OH D_3 as compared to vitamin D_3 for poultry. However, in humans it is reported that 0.025 µg of vitamin D equals 1 IU of vitamin D activity, whereas 0.005 µg 25-OH D_3 equals 1 IU of vitamin D activity (Standing Committee on the Scientific Evaluation of Dietary Reference Intake, et al., 1997). If similar relative activities apply for poultry,

3.125 µg/kg of vitamin D (125 IU/kg) fed to broiler breeders would be considered to be vitamin D deficient, as the NRC recommended vitamin D level for egg-laying birds is 300 IU (National Research Council, 1994). When hatching eggs are deficient or low in vitamin D, embryonic mortality and/or chick deformation increases (Sunde, et al., 1978; Stevens, et al., 1984; Elaroussi, et al., 1993). Therefore, in the Antencio (2005) study, the reason for the greater embryonic mortality of eggs from the vitamin D_3 hens is most likely due to the insufficient vitamin D activity to support normal embryonic development, while the greater amount vitamin D activity given to the 25-OH D_3 fed hens was able to support normal embryonic development. This is further supported by the fact that when the dose of 25-OH D_3 and vitamin D was increased to 12.5 µg/kg there was no difference in embryonic mortality (Atencio, et al., 2005). In the current study, both groups of hens received well over the NRC recommended level of vitamin D activity (2760 IU) for egg-laying hens and therefore eggs would be expected to have sufficient levels of vitamin D metabolites to support normal embryonic development. However, even when vitamin D is fed at sufficient levels, the addition of 1,100 ICU of 25-OH D_3 to a diet that already contained 2,200 ICU of vitamin D_3 increased percent hatch of fertile turkey eggs, with a % hatchability of 37.5 % compared to 19.7 % for the eggs from the turkeys not supplemented with 25-OH D_3 (Manley, et al., 1978). That study did not report at what stage of embryo development the losses occurred so a direct comparison of time of embryonic death with the present study is not possible. In addition, previous work in our lab has shown broiler breeder supplementation of 25-OH D_3 to significantly reduce early embryonic mortality as compared to vitamin D_3 (Chapter 6; Table 6-2). There appears to be some protective effect of 25-OH D_3 during early

embryonic development. Further research into the means of this protection is needed as this has not been shown in previous work.

7.3.4. *Effect of Maternal Dietary Vitamin D Source on Breeder and Chick Plasma 25-OH D_3 and Early Chick Growth*

Plasma 25-OH D_3 levels of broiler breeders at 64 weeks of age was significantly greater for the broiler breeders that were consuming 25-OH D_3 (P=0.0030; Figure 7-4), with an average plasma 25-OH D_3 level of 60.54 ± 5.72 ng/ml plasma as compared to 38.44 ± 3.60 ng/ml for the birds on the Control treatment (Figure 7-4). Mitchell et al. (1997) also reported increases in plasma 25-OH D_3 when dietary 25-OH D_3 was provided to broilers. These results indicate that dietary 25-OH D_3 is effective at increasing circulating plasma levels of 25-OH D_3 within the broiler breeder. Previous work has shown that the vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 content within the egg yolk (Mattila, et al., 1999). Therefore, with the increased amount of plasma 25-OH D_3 of the broiler breeders on the 25-OH D_3 diet, it is probable that more 25-OH D_3 would get passed into the egg yolk.

The plasma 25-OH D_3 levels of day-old chicks were not different due to maternal treatment for the chicks from the 31 to 33 wk old breeders (Table 7-7). Interestingly, there was a trend toward a greater level of plasma 25-OH D_3 level of the chicks from the Control treatment, for the broiler breeder age of 46 to 48 wk (P=0.0564; Table 7-7). However, at a breeder age of 61 to 63 wk, the maternal 25-OH D_3 chicks had a significantly greater level of plasma 25-OH D_3 (Table 7-7). Surprisingly, plasma 25-OH D_3 levels at 4 d post-hatch were greater in the chicks from the Control maternal treatment

at breeder ages of 31 to 33 and 46 to 48 wk (Table 7-7). These results indicate that the maternal effect on broiler plasma 25-OH D_3 is transient, however the reason that plasma 25-OH D_3 would be greater for the Control than the 25-OH D_3 maternal treatment, in 4 d old chicks from broiler breeder ages of 31 to 33 and 46 to 48 wk, is not known and requires further investigation. Previous research (Chapter 4; Figure 4-1), clearly showed that direct dietary 25-OH D_3 supplementation significantly increased plasma circulating levels of this metabolite in broiler chicks. However, elevated broiler breeder plasma 25-OH D_3 does not appear to affect chick plasma 25-OH D_3.

Decreases in plasma 25-OH D_3 levels have previously been observed to occur in the chick after 1 d of age when chicks received only dietary vitamin D_3 for the first 10 d post-hatch (see chapters 4 and 6). In the current study this same trend was observed in broilers for broiler breeder ages 31 to 33 and 46 to 48 but not at the late broiler breeder age of 61 to 63 wk. To the authors' knowledge, no one has compared the effect of vitamin D source in the breeder diet on chick levels of 25-OH D_3. Although it has been established that dietary 25-OH D_3 has a positive effect on plasma 25-OH D_3 of the bird to which it is being fed (Yarger, et al., 1995), the effect of maternal nutritional has not been reported. In future work, it will be beneficial to assess whole body 25-OH D_3 rather than peripheral 25-OH D_3 in the chick, as vitamin D_3 can be stored in the body (Mawer, et al., 1972). In rats, vitamin D_3 has been shown to be stored mainly in adipose tissue but has also been found to be stored in the kidney and liver (Rosenstreich, et al., 1971).

There was a significant interaction effect of broiler breeder age and dietary treatment on plasma 25-OH D_3 levels at 1 d post-hatch (P=0.0018; SEM=4.11; Figure 7-5A). Chicks from broiler breeder ages 46 to 48 wk had a significantly greater plasma 25-

OH D_3 than those from the early hatch of 31 to 33 wk old broiler breeders. For the vitamin D treatment the chicks from the 46 to 48 wk broiler breeders also had a great plasma 25-OH D_3 than chicks hatch from 61 to 63 wk broiler breeders, while there was no difference for the 25-OH D_3 treatment. At 4 d post hatch, broiler breeder age had a significant effect on plasma 25-OH D_3 ($P<0.0001$; SEM=4.00), with the chicks from the late hatch (61 to 63 wk) having the greatest plasma 25-OH D_3 level (Figure 7-5B).

There were no significant effects of maternal dietary vitamin D_3 source on chick growth and feed efficiency to 7 days for any of the maternal broiler breeder ages (Table 7-8). This is in agreement with previous work in our lab that found no difference in 7 d BW, gain and feed conversion of chicks from broiler breeders fed 34.5µg of 25-OH D_3 per L of water 25-OH D_3 was given in addition to 3,000 IU of dietary vitamin D_3 (Chapter 6; Table 6-4).

7.3.5. *Effect of Maternal Dietary Vitamin D Source on Early Chick Innate Immune Function*

There was no difference in the percentage of phagocytising leukocytes as well as no difference in the number of *E. coli* engulfed per cell at either 1 or 4 d post-hatch for the breeder flock ages of 31-33 and 46-48 (Table 7-9). Although there were no differences between treatments in the percentage of cells phagocytosing *E. coli*, there was an increase in mean fluorescence at 1 d post hatch of broilers from 61 to 63 wk old broiler breeders (Table 7-9). The increase fluorescence indicates a greater number of bacteria being engulfed per cell thereby increasing the potential for the bacteria to be destroyed. Although not directly comparing 25-OH D_3 and vitamin D_3, previous studies

have shown that when chicks were deficient in vitamin D, phagocytic potential of macrophages to sheep red blood cells *in vitro* was reduced (Aslam, et al., 1998). Vitamin D-deficient mice have reduced macrophage phagocytic capability to yeast (Bar-Shavit, et al., 1981). Therefore, the results of the current study indicate that maternal dietary 25-OH D_3 supports chick phagocytic immune function at least as well (and in some cases better) than vitamin D when fed at adequate levels.

The percent of leukocytes phagocytising at least 1 bacterium was greatest for chicks from the mid hatch (47 to 48 wk) at both 1 ($P<0.0001$; SEM=1.58; Figure 7-6A) and 4 d ($P=0.0003$; SEM=1.84; Figure 7-6B) post-hatch. In addition, at 1 d post-hatch, percent phagocytosis was lowest in chicks from the 31 to 33 wk old broiler breeders (Figure 7-6A), while at 4 d post hatch there was no difference between the early and late hatch (Figure 7-6B). There was a significant interaction of broiler breeder age and dietary treatment on the amount of bacteria phagocytosed per leukocyte ($P=0.0152$; SEM=84.37; Figure 7-7A). Chicks from the mid broiler breeder age of 46 to 48 wk had the greatest amount of fluorescence per cell, while chicks from the early broiler breeder age had the lowest mean fluorescence. Cells from chicks hatched from the late broiler breeder age phagocytosed a fewer number of bacteria per cell than those cells of chicks from the mid-hatch; however cells from chicks on the vitamin D_3 treatment was lower than the 25-OH D_3 chicks at this age. At 4 d post hatch fluorescence was greatest for the chicks from the mid hatch (47 to 48 wk) and lowest for the chicks from the early hatch (31 to 33 wk; Figure 7-7B).

There were no significant differences among treatments due in oxidative burst of chicks at 1 d post hatch from 5 to 15 minutes post-stimulation of chick cells at the

breeder age of 31-33 wk (Figure 7-8A). There was greater oxidative burst response at 20 minutes post-stimulation for the Control birds than the 25-OH D_3 birds (Figure 7-8A). At 4 d post-hatch there were no treatment effects on the heterophil oxidative burst response (Figure 7-8A). However, for chicks from the breeders at 46 to 48 wk, the oxidative burst at 1 d post-hatch was significantly greater at 10, 15 and 20 min post-stimulation for the maternal 25-OH D_3 chicks than the Control chicks (Figure 7-8B). At this same breeder age, the chicks at 4 d post-hatch exhibited no treatment effect on oxidative burst response until 20 minutes post-stimulation at which time the Control chicks had a significantly greater oxidative burst than the 25-OH D_3 chicks (Figure 7-8B). At the broiler breeder age of 61 to 63 wk there were no significant differences between treatments in the oxidative burst response at either 1 or 4 d post-hatch (Figure 7-8C).

At 20 min post-stimulation, the 1 d post-hatch chick heterophils from the late hatch (61 to 63 wk) had the lowest oxidative burst index, while there was no difference between the chick heterophils from early and mid hatches (Figure 7-9A). However, at 4 d post-hatch the chick heterophils from the mid hatch (46 to 48 wk) had the lowest oxidative burst, while there was no difference between the chick heterophils from the early and late hatches (Figure 7-9B)

Leukocytes from chicks in the maternal 25-OH D_3 treatment consistently killed more *E. coli* than those from the Control group at all broiler breeder and chick ages, except at d 1 post-hatch of the 46 to 48 wk broiler breeder age (Table 7-10). There is no killing data presented for 1 d post-hatch at the broiler breeder age of 31 to 33 wk as the bacteria was not available. At 1 d post-hatch, there was no difference in the percentage of *E. coli* killed by the leukocytes of the mid and late hatch chicks (Figure 7-10A). At 4

d post-hatch, chicks from the early hatch (31 to 33 wk) had the greatest percentage of killed bacteria while the chicks from the 61 to 63 wk hatch had the lowest ($P<0.0001$; SEM=3.95; Figure 7-10B).

The potential of maternal dietary 25-OH D_3 to alter immune function of the chick may be due to the fact that 25-OH D_3 is transported into the fertile egg and may affect the embryo 25-OH D_3 status (Sunde, et al., 1978; Soares, et al., 1995). In the current study, the major indicator that maternal dietary 25-OH D_3 altered the innate immune function of the broiler chick was increased bactericidal capability of the 25-OH D_3 chicks (Table 7-10). Huff et al. (2000) reported that supplementation of 2,064 IU vitamin D_3 per L of drinking water for the first 5 d of brooding and with 4,128 IU/L to turkeys after weekly weighing and bacterial challenges in addition to 2,204 IU dietary vitamin D_3 increased resistance to repeated bacterial infections and lowered heterophil:lymphocyte ratios as compared to birds exposed to the same challenge but did not receive any additional vitamin D_3. Limited research has been done on supplementation of vitamin D above the requirement on immune function, however, a vitamin D deficiency in the chicken reduces both cellular and innate immune responses (Aslam, et al., 1998). The effect of vitamin D metabolites on cells of the innate immune system is in part due to the fact that humans and rodent phagocytic cells have been shown to be capable of metabolizing 25-OH D_3 to $1,25(OH)_2D_3$ (Gray, et al., 1982; Cohen and Gray, 1984). Although there is limited research with poultry on the effects of vitamin D metabolites on the bactercidal capability of innate immune cells, recent research with cultured human neutrophils and macrophages has shown that $1,25(OH)_2D_3$ stimulates antimicrobial activity through the increased expression of antimicrobial peptide genes (Wang, et al., 2004; Gombart, et al.,

2005). Although the current study did not investigate molecular mechanisms of the enhanced bactericidal capability observed in the 25-OH D_3 chicks, this could explain the difference in bacteria killing capability while little differences in phagocytosis were observed between the maternal treatments. If the *ex vivo* results hold true *in vivo*, than this may result in less resources used by the bird to fight an infection and therefore a reduction in the metabolic consequences of the inflammatory response.

The current study looked at the strength of the oxidative burst response of cells from the young chick. The most significant effect of maternal 25-OH D_3 was observed in cells from day old chicks from the broiler breeder age of 46 to 48 wk, where the cells of the 25-OH D_3 chicks had a greater response just 10 min after stimulation (Figure 7-3B). This could be important to the chick *in vivo* as have a greater capacity to kill bacteria at just one day post-hatch could protect the chick from potential pathogens when they are first place in the barn. To the authors' knowledge, no previous studies have investigated the effect of vitamin D source on heterophil oxidative burst response. However, Fritts et al., (2004) reported that nitric oxide production of macrophages from broilers was not affected by either source or level of vitamin D_3 or 25-OH D_3.

Broiler breeder age also significantly affected early chick innate immune function. It appears that chicks hatching from mid production broiler breeders had improved innate immune function, while those from the early production hatch had the lowest. The effect of broiler breeder age in early chick innate immune function has not been previously reported. However, egg hatchability has been previous reported to be the greatest at mid production (43 wk) broiler breeder ages, and lowest at a late production age (57 wk) (Suarez, et al., 1997; O'Dea, et al., 2006). In addition, embryonic mortality

and culls of chicks at hatch were lowest in eggs from mid production age broiler breeders (Suarez, et al., 1997; O'Dea, et al., 2006). When chicks from early, mid and late broiler breeders were grown out, broiler chick BW at 42 days was lowest in chicks from young broiler breeders (O'Dea, et al., 2006). Although these effects are not directly related to immune function of the chick, it is interesting how they all follow the same pattern of response. Broiler breeder age therefore appears to be a determining factor in innate immune function of the broiler chick.

The results of this research indicate that maternal dietary 25-OH D_3 increased some aspects of innate immune function. Increased bactericidal activity of leukocytes from the 25-OH D_3 chicks at all broiler breeder and chick ages studied, as well as increased phagocytic and oxidative burst response at some ages measured indicate that the immune system in these young chicks is potentially more mature and better equipped to handle an infectious challenge. In addition, 25-OH D_3 appears to have some protective effect on early embryonic survival, resulting in a greater % hatch, which has not been previously reported.

7.4. REFERENCES

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Table 7-1. Effect of supplemental dietary vitamin D source on broiler breeder hen day settable egg production

Treatment	N	#Eggs/bird from 25 to 40 wk	# Eggs/bird from 41 to 64 wk	Total # of Eggs/bird 25 to 64
Vitamin D_3[1]	2	78	102	185
25-OH D_3[2]	2	77	104	186
Pooled SEM		1.7	2.1	3.5
ANOVA			Probabilities	
Treatment		0.6538	0.5263	0.8368

[1]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.
[2]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.
[3]N of 4 is the number of pens of broiler breeders with approximately 24 breeder hens per pen.

Table 7-2. Effect of dietary vitamin D source on broiler breeder egg quality from 29 to 64 wk of age

Treatment	N	Egg Wt (g)	SG[1]	% Yolk[1]	% Albumen[1]	Albumen height[1] (mm)	% Shell[1]	Shell Thickness[1] (mm)
					29 wk			
Vitamin D_3[2]	2	53.93	1.086	27.43	60.67	8.3	9.57	0.355[b]
25-OH D_3[3]	2	54.07	1.087	27.55	59.55	8.5	9.76	0.384[a]
SEM		0.26	0.001	0.29	0.47	0.07	0.16	0.001
ANOVA	DF				Probabilities			
Treatment	1	0.7315	0.4192	0.7970	0.0962	0.2083	0.5166	0.0011
					36 wk			
Vitamin D_3	2	59.87	1.081	29.58	57.17	6.67	8.74	0.339
25-OH D_3	2	60.70	1.083	29.76	56.81	7.29	9.01	0.388
SEM		0.69	0.001	0.27	0.36	0.16	0.09	0.028
ANOVA	DF				Probabilities			
Treatment	1	0.4854	0.2796	0.6844	0.5567	0.1082	0.1921	0.3424
					42 wk			
Vitamin D_3	2	63.03	1.080	31.00	56.17	6.67	8.61	0.311
25-OH D_3	2	63.07	1.081	31.14	55.33	7.11	8.79	0.326
SEM		0.70	0.001	0.21	0.31	0.12	0.10	0.004
ANOVA	DF				Probabilities			
Treatment	1	0.9625	0.3348	0.6855	0.1914	0.1283	0.3473	0.1367
					46 wk			
Vitamin D_3	2	62.96	1.080	31.67	55.56	6.37	8.66	0.332
25-OH D_3	2	63.53	1.080	31.51	55.13	6.88	8.76	0.336
SEM		1.05	0.001	0.07	0.48	0.15	0.11	0.004
ANOVA	DF				Probabilities			
Treatment	1	0.7372	0.7274	0.2690	0.5923	0.1396	0.5798	0.4798
					52 wk			
Vitamin D_3	2	65.85	1.08	32.22	59.13	6.01	8.84	0.331
25-OH D_3	2	66.46	1.08	31.92	59.23	6.65	8.70	0.319
SEM		1.10	0.001	0.26	0.51	0.13	0.21	0.005
ANOVA	DF				Probabilities			
Treatment	1	0.7322	0.7542	0.4927	0.8946	0.0696	0.6918	0.2765
					58 wk			
Vitamin D_3	2	67.42	1.079	31.99	59.58[a]	5.33[b]	8.51	0.347
25-OH D_3	2	69.74	1.080	32.43	58.64[b]	6.11[a]	8.65	0.362
SEM		1.08	0.0004	0.30	0.15	0.11	0.12	0.007
ANOVA	DF				Probabilities			
Treatment	1	0.2697	0.2727	0.0915	0.0494	0.0398	0.4996	0.2923
					64 wk			
Vitamin D_3	2	68.97	1.077	31.96	59.54	5.29	8.43	0.316
25-OH D_3	2	69.14	1.078	32.86	58.63	6.84	8.54	0.319
SEM		2.00	0.001	0.40	0.41	0.63	0.17	0.004
ANOVA	DF				Probabilities			
Treatment	1	0.9576	0.7319	0.2542	0.2595	0.2241	0.6981	0.5441

[a,b]Treatment means within a column and breeder age with different superscripts are significantly different.

[1]Specific gravity was measured by the floatation method with a series of saline solutions of increasing specific gravity ranging from 1.060 to 1.010 in increments of 0.002. Shell wt = weight of washed and air-dried egg shell (with membrane). Shell thickness was determined on the eggshell from the middle of the egg using a micrometer. Percent shell, yolk and albumen were determined as a percentage of the total egg weight.

[2]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. [3]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

Table 7-3. Effect of dietary vitamin D source on bone mineral density of broiler breeders from 21 to 65 wk of age

Treatment	N	Femur Density (mg/cm^3)			Femur Cross-Sectional Area (mm^2)		
		Total[1]	Cortical[2]	Trabecular[3]	Total	Cortical	Trabecular
				21 wk[4]			
Initial Scan	12	487.68	984.36	52.13	39.82	17.84	20.28
SE		12.37	8.52	3.69	1.64	0.58	1.29
				32 wk			
Vitamin D_3[5]	8	527.68	1016.82	48.27	43.88	20.93	21.08
25-OH-D_3[6]	8	527.46	1008.94	47.16	43.63	20.89	20.95
SE		10.50	4.21	2.08	1.08	0.31	0.93
ANOVA				Probabilities			
Treatment		0.8301	0.1956	0.7097	0.8666	0.9289	0.9215
				52 wk			
Vitamin D_3	8	515.11	984.63	86.18	44.95	20.85	22.07
25-OH-D_3	8	514.26	982.29	75.16	43.51	20.51	21.41
1.02		11.30	7.59	4.19	1.21	0.31	1.02
ANOVA				Probabilities			
Treatment		0.9676	0.8287	0.0731	0.4045	0.4391	0.6490
				65 wk			
Vitamin D_3	8	555.45	1044.42	87.57	44.60	21.04	21.44
25-OH-D_3	8	550.36	1035.07	88.68	44.07	20.74	20.99
SE		13.11	11.49	6.69	1.20	0.28	1.02
ANOVA				Probabilities			
Treatment		0.7819	0.5635	0.9066	0.7519	0.4542	0.7525

[1]Total = the total for the entire bone.
[2]Cortical = measurements taken on the area define as >500mg/cm^3 and the outer part of the bone.
[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.
[4]Bone scans were conducted prior to start of experimental treatments, therefore no treatment effect.
[5]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.
[6]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

Table 7-4. The effect of dietary vitamin D source on broiler breeder carcass characteristics and ovarian morphology.

Treatment	N	Live Wt (g)	Breast weight (% of live wt)	Pectoralis major (% of live wt)	Pectoralis Minor (% of live wt)	Fat Pad (% of live wt)	Liver (% of live wt)	Spleen (g)	Ovary (g)	Oviduct (g)	Stroma (g)	LYF[3] (#)	SYF[4] (#)	Atretic YF[5] (#)
Vitamin D_3[1]	48	3415	16.92	13.33	3.59	2.68	1.70	3.26	55.37	61.36	9.82	4.31	13.56	15.24
25-OH-D_3[2]	43	3465	16.80	12.81	3.94	2.78	1.68	3.13	58.77	64.87	10.01	4.53	12.42	16.44
SE		0.07	0.31	0.28	0.10	0.19	0.04	0.19	3.43	2.86	0.39	0.24	0.73	2.14
ANOVA							Probabilities							
Treatment		0.6703	0.7828	0.1999	0.1353	0.7365	0.6522	0.6464	0.4975	0.4074	0.7329	0.5262	0.2924	0.7027

[1]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.
[2]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.
[3]Large yellow follicle (>10 mm diameter).
[4]Small yellow follicle (5 to 10 mm diameter).
[5] Atretic follicles >5 mm in diameter.

Table 7-5. Effect of maternal vitamin D source on hatchability and fertility from 30 to 32, 47 to 49, and 61 to 63 wk-old broiler breeders

Treatment		% Fertility[1]	% Hatch[1] (total eggs)	% Hatch[1] (fert. Eggs)	% Early Mortality[1]	% Late Mortality[1]	% Total Mortality	% IPL[1]	% IPD[1]	% EPL[1]	% EPD[1]	% Dead[1]	% Cull[1]
	N						**31 to 33 wk**						
Vitamin D_3[2]	49	91.88[a]	70.64	76.28	4.49	22.91	27.41[a]	1.18	0.45	0.99	-	-	0.01
25-OHD_3[3]	47	85.62[b]	65.90	76.97	6.61	11.82	18.43[b]	0.79	0.79	0.41	-	-	0.21
Chi Square	DF						Probabilities						
Treatment	1	0.0110	0.1635	0.9874	0.2698	0.1545	0.0024	0.8634	0.2188	0.4981	-	-	0.9795
							46 to 48 wk						
Vitamin D_3	34	92.04	85.60	92.84	3.33	1.64	4.97	-	0.49	0.01	0.01	0.21	0.01
25-OHD_3	35	94.24	82.02	87.18	4.31	2.74	7.04	-	0.01	0.88	0.20	0.01	0.76
Chi Square	DF						Probabilities						
Treatment	1	0.3020	0.3814	0.0649	0.3248	0.2275	0.1200	-	0.9803	0.9806	0.9680	0.9803	0.9806
							61 to 63 wk						
Vitamin D_3	26	91.09	78.38[b]	86.23[b]	5.05	4.31	9.35	-	0.27	0.87	0.88	0.56	0.25
25-OHD_3	25	92.56	84.62[a]	91.07[a]	3.47	3.24	6.72	-	0.01	1.37	0.01	0.01	0.55
Chi Square	DF						Probabilities						
Treatment	1	0.3503	0.0217	0.0291	0.4200	0.2430	0.2226	-	0.9724	0.9500	0.9832	0.9724	0.5425

[a,b]Treatment means within same column with different superscripts are significantly different ($P<0.05$).

[1]% Fertility = % of set eggs that were fertile; % Hatch = % of chicks that hatch from all set eggs; % hatch (fert. Eggs) = % of chicks that hatched from all fertile eggs; % Early Mortality= % embryonic mortality of fertile eggs from 0 to 7 d; % Late Mortality = % embryonic mortality of fertile eggs from 8 to 18 d; % Late Hatch = % of chicks from fertile eggs taking longer than 21.5 days to hatch; %IPL = % internal pip live of fertile eggs (chick pips through only membrane and is alive) ; % IPD= % internal pip dead of fertile eggs (chick pips through only membrane and is dead); % EPL= % external pip live of fertile eggs (chick pips through shell and is alive); % EPD= % external pip dead of fertile eggs (chick pips through shell and is dead); % dead= % of dead chicks at hatch of fertile eggs; % cull= % of chicks culled at hatch of fertile eggs.

[2]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

Table 7-6. Effect of maternal vitamin D source on set, transfer and weight loss of hatching eggs during incubation and chick hatch BW

Treatment	Set Egg Weight[1] (g)	Transfer Egg Weight[1] (g)	% Weight Loss[1]	Eggshell Conductance[1] (mg H_2O/d/mm Hg)	Chick BW (g)
			31 to 33 wk		
Vitamin D_3[2]	54.41	47.87	12.25	10.64	37.54[b]
25-OHD_3[3]	54.53	48.24	12.94	10.34	38.17[a]
SEM	0.186	0.183	0.406	0.201	0.198
ANOVA			Probabilities		
Treatment	0.6544	0.1575	0.2360	0.2873	0.0293
			46 to 48 wk		
Vitamin D_3	63.69	56.39	11.74	14.64	44.10
25-OHD_3	64.39	57.00	11.61	14.63	44.21
SEM	0.283	0.296	0.184	0.449	0.266
ANOVA			Probabilities		
Treatment	0.0863	0.1520	0.6177	0.9863	0.7416
			61 to 63 wk		
Vitamin D_3	67.66	58.90	13.14	13.81	46.17
25-OHD_3	68.26	59.41	13.13	13.87	46.05
SEM	0.348	0.328	0.19	0.717	0.335
ANOVA			Probabilities		
Treatment	0.2385	0.2796	0.9598	0.8896	0.7955

[a,b] Treatment means within same column with different superscripts are significantly different ($P<0.05$).

Set egg weight = weight of egg when first put in incubator; transfer egg weight = weight of egg after 18 d of incubation; % weight loss = % of weight loss of the egg from 0 to 18 d of incubation; eggshell conductance = rate of water loss from egg when stored for 7 d covered with desiccant.

[2]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

Table 7-7. Effect of maternal vitamin D source on broiler plasma 25-OH-D_3 at 1 d post-hatch from broiler breeders at ages 31 to 33, 46 to 48, and 61 to 63 wk of age.

Maternal Treatment		Plasma 25-OH-D_3 (ng/ml) 1 d	4 d
		31-33 wk[1]	
	Vitamin D_3[2]	15.95 (21)[4]	12.91[a] (27)
	25-OH-D_3[3]	16.10 (18)	10.38[b] (28)
SE		0.86	0.67
ANOVA		Probabilities	
Treatment		0.9011	0.0100
		46-48 wk	
	Vitamin D_3	38.74 (20)	12.36[a] (25)
	25-OH-D_3	27.33 (13)	7.98[b] (27)
SE		4.31	1.41
ANOVA		Probabilities	
Treatment		0.0564	0.0272
		61-63 wk	
	Vitamin D_3	25.94[b] (26)	55.38 (28)
	25-OH-D_3	36.40[a] (27)	50.72 (29)
SE		3.40	
ANOVA		Probabilities	
Treatment		0.0345	0.7129

a-b; Means within the same column and maternal age with different superscripts are significantly different ($P<0.05$).

[1]Broiler breeder age.

[2]Chicks from broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Chicks from broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[4]Treatment mean followed by n in parentheses.

Table 7-8. Effect of maternal vitamin D source on one week broiler growth and production efficiency

	Hatch BW	7 d BW	0 to 7 d Gain	0 to 7 d Feed Consumption (g/bird/d)	FCE
Treatment[1]			**31 to 33 wk**		
Vitamin D_3[2]	38.03	118.04	11.49	13.12	1.14
25-OHD_3[3]	38.05	112.75	10.66	12.68	1.21
SEM	0.390	2.767	0.719	0.568	0.048
Sex					
Female	38.03	116.92	10.68	12.48	1.19
Male	38.50	113.87	11.46	13.32	1.17
SEM	0.361	2.916	0.759	0.599	0.050
ANOVA			Probabilities		
Treatment	0.9679	0.2315	0.4615	0.6240	0.3678
Sex	0.3610	0.4820	0.4852	0.3492	0.7694
Treatment X Sex	0.9464	0.4855	0.4439	0.8612	0.2266
Treatment			**46 to 48 wk**		
Vitamin D_3	43.84	142.94	13.70	15.99	1.17
25-OHD_3	44.13	142.06	13.74	16.49	1.20
SEM	0.190	4.630	0.746	0.813	0.025
Sex					
Female	43.74	142.78	13.96	16.50	1.19
Male	44.23	142.22	13.48	15.97	1.19
SEM	0.180	4.414	0.711	0.813	0.024
ANOVA			Probabilities		
Treatment	0.2956	0.8950	0.9712	0.6708	0.4020
Sex	0.0857	0.9319	0.6518	0.6474	0.9925
Treatment X Sex	0.0555	0.6353	0.6442	0.7494	0.0605
Treatment			**61 to 63 wk**		
Vitamin D_3	43.50	182.55	18.88	15.21	0.80
25-OHD_3	45.99	186.01	18.77	16.98	0.90
SEM	1.63	3.33	0.577	1.210	0.051
Sex					
Female	43.16	182.70	19.18	16.41	0.86
Male	46.34	185.85	18.47	15.80	0.85
SEM	1.63	3.33	0.577	1.210	0.051
ANOVA			Probabilities		
Treatment	0.3001	0.4764	0.8974	0.3218	0.1761
Sex	0.1912	0.5164	0.3984	0.7251	0.9324
Treatment X Sex	0.3061	0.8668	0.7242	0.3573	0.1860

[1]n = 12 pens per treatment.

[2]Broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

Table 7-9. Effect of maternal dietary vitamin D source on *in vitro* innate immune cell phagocytosis of *E. coli* at 1 and 4 days post-hatch from broiler breeders at 31 to 33, 46 to 48, and 61 to 63 wk of age

	% of Cells Phagocytising		Mean Fluorescence	
Maternal Treatment	1 d	4 d	1 d	4 d
			31-33 wk[1]	
Vitamin D_3[2]	36 (19)[4]	50 (22)	1381 (19)	1365 (22)
25-OH D_3[3]	33 (19)	44 (21)	1441 (19)	1302 (21)
SE	1.9	2.3	41.0	28.1
ANOVA			Probabilities	
Maternal Treatment	0.0839	0.0898	0.2952	0.1181
			46-48 wk	
Vitamin D_3	50 (25)	54 (24)	2837 (25)	2618 (24)
25-OH D_3	49 (25)	51 (27)	2718 (25)	2711(27)
SE	1.4	1.5	76.1	80.6
ANOVA			Probabilities	
Maternal Treatment	0.9687	0.2484	0.2765	0.4021
			61-63 wk	
Vitamin D_3	44 (26)	47 (27)	2011[b] (26)	1996 (27)
25-OH D_3	47 (26)	46 (28)	2223[a] (26)	1964 (28)
SE	1.5	1.4	60.8	56.3
ANOVA			Probabilities	
Maternal Treatment	0.1057	0.6625	0.0171	0.6916

[a-b.] Means within the same column and maternal age with different superscripts are significantly different ($P<0.05$).

[1]Broiler breeder age (weeks).

[2]Chicks from broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Chicks from broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[4]Treatment mean followed n in parentheses.

Table 7-10. Effect of maternal dietary vitamin D source on innate immune cell killing of *E.coli* at 1 and 4 days post-hatch from broiler breeders at 31 to 33, 46 to 48, and 61 to 63 wk of age

Maternal Treatment		% E. coli Killed	
	n	1 d	4 d
		31-33 wk[1]	
Vitamin D_3[2]	15	-[4]	80[b]
25-OH D_3[3]	15	-	88[a]
Chi Square		Probabilities	
Treatment		-	<0.0001
		46-48 wk	
	n		
Vitamin D_3	30	55	72[b]
25-OH D_3	30	52	74[a]
Chi Square		Probabilities	
Treatment		0.6679	0.0352
		61-63 wk	
	n		
Vitamin D_3	30	51.[b]	51[b]
25-OH D_3	30	54[a]	57[a]
Chi Square		Probabilities	
Treatment		<0.0001	<0.0001

[a,b] Means within the same column and maternal age with different superscripts are significantly different ($P<0.05$).

[1] Broiler breeder age.

[2]Chicks from broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[3]Chicks from broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age.

[4]Data not available for 1 d post-hatch at broiler breeder age of 31 to 33 wk as bacteria was not available at the time of sample collection.

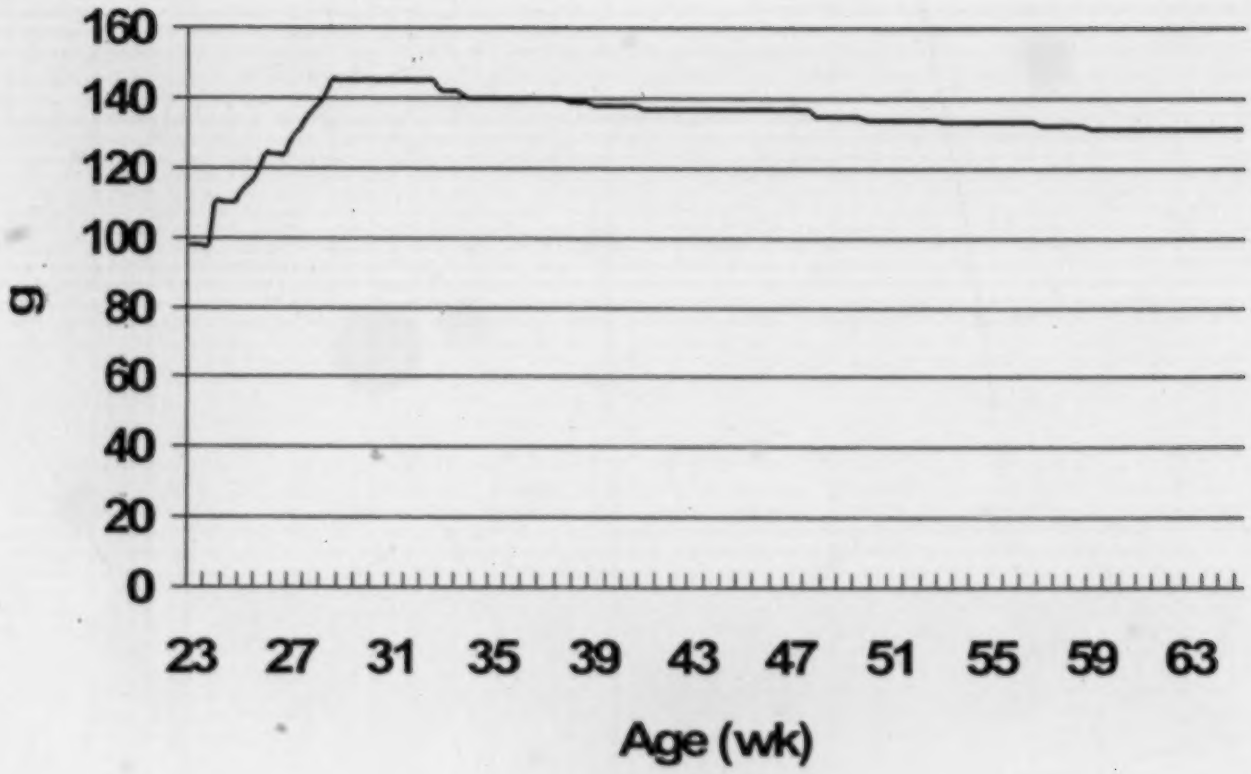


Figure 7-1. Feed allocation to broiler breeders from 23 to 65 wk.

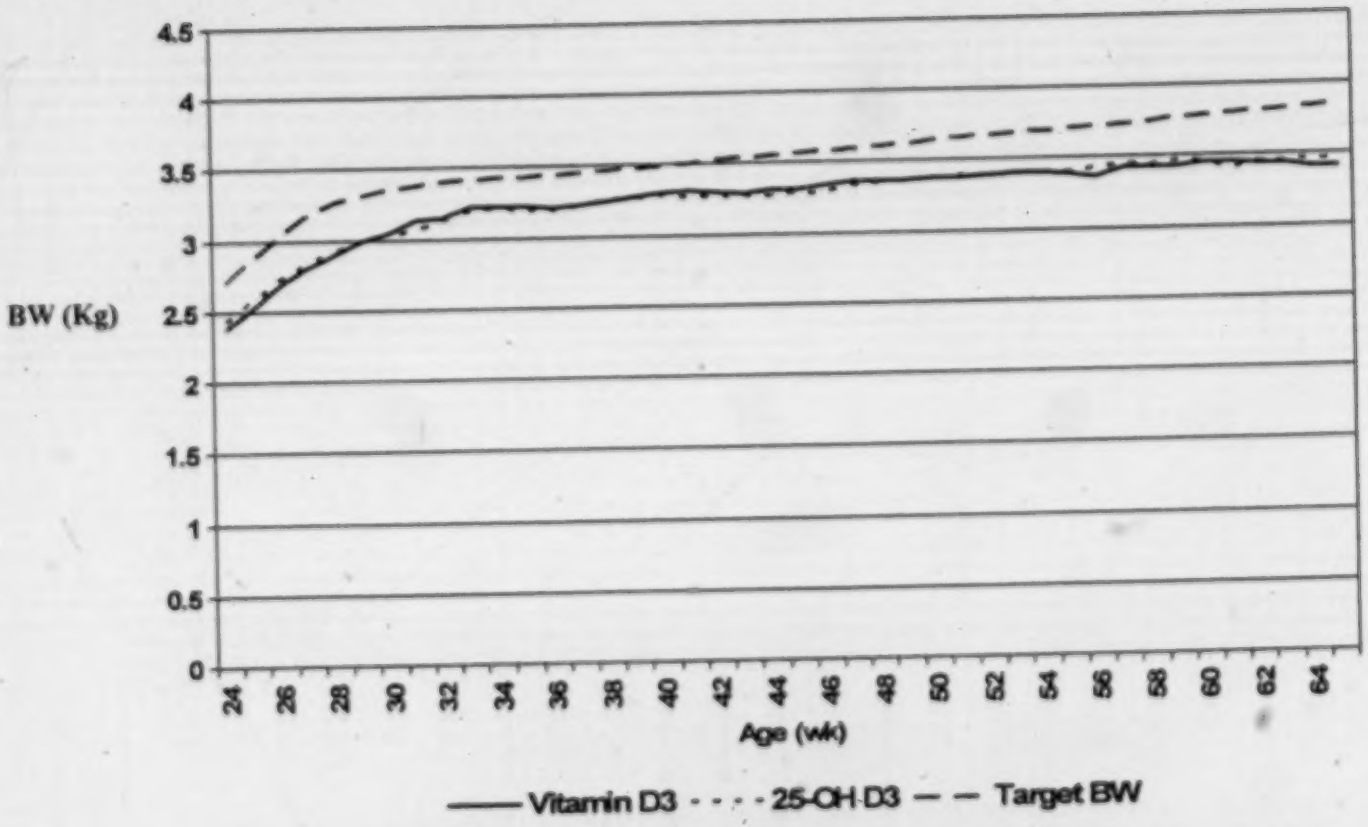


Figure 7-2. Effect of dietary vitamin D source on broiler breeder BW from 24 to 65 wk.
The Control treatment was broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 was broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. n= 4 pens of broiler breeders (2 per treatment) containing approximately 24 broiler breeder hens per pen.

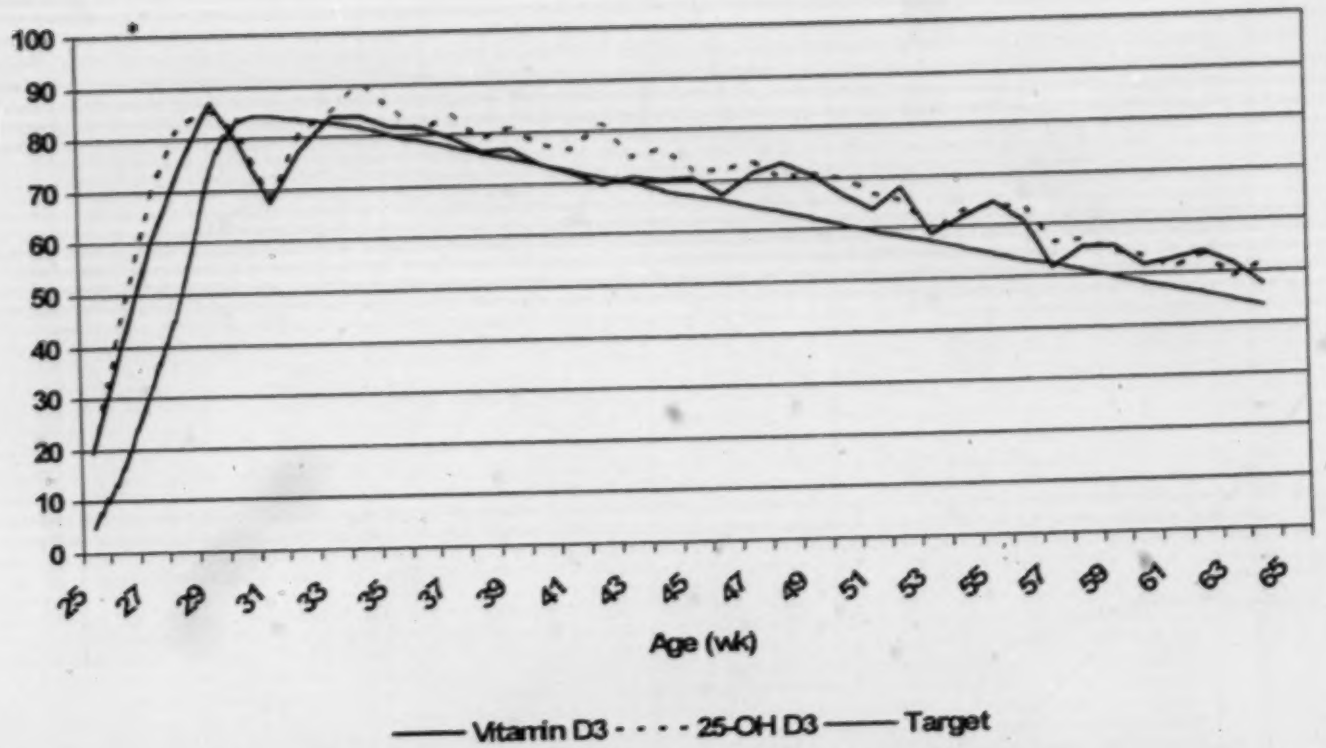


Figure 7-3. Effect of vitamin D source on % settable egg production.
The Control treatment was broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 was broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. n= 4 pens of broiler breeders (2 per treatment) containing approximately 24 broiler breeder hens per pen. Significant differences are indicated with an asterisk (*).

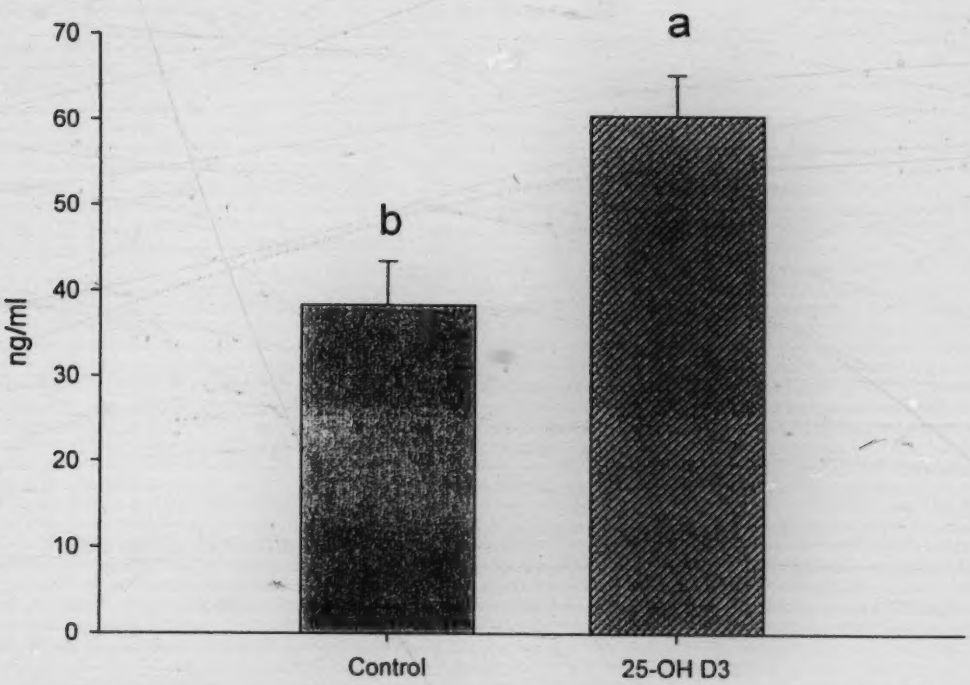


Figure 7-4. The effect of vitamin D source on broiler breeder plasma 25-OH D_3. The Control treatment was broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 was broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. n= 4 pens of broiler breeders (2 per treatment) containing approximately 24 broiler breeder hens per pen. Significant differences are indicated with a differing lowercase letter.

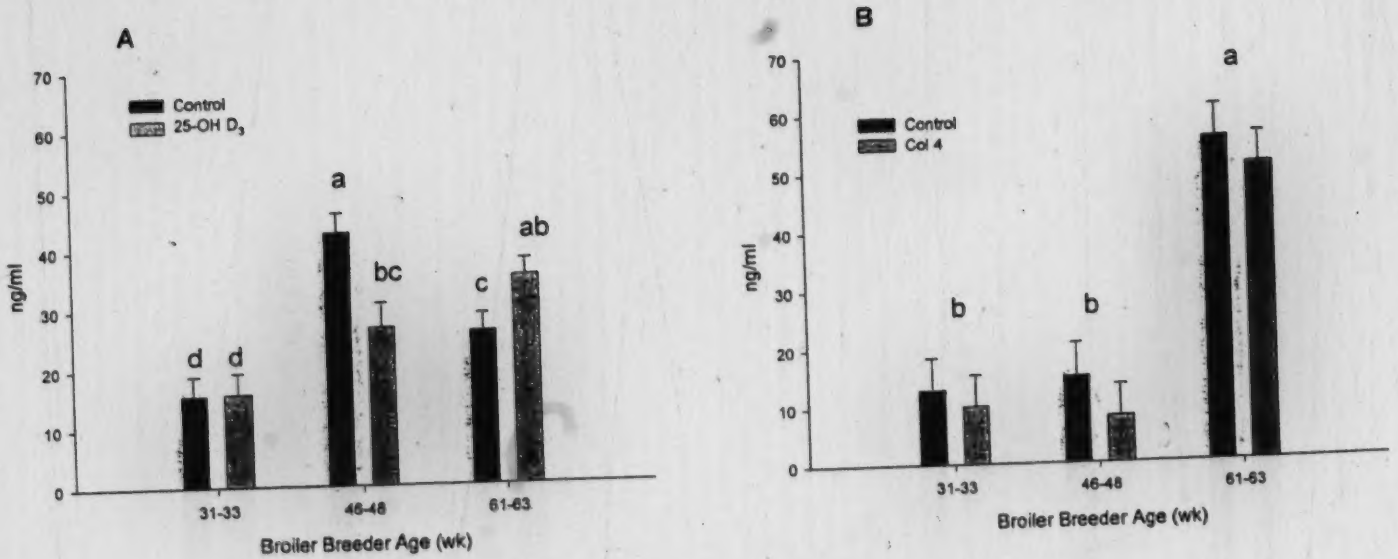


Figure 7-5. The effect of broiler breeder age on chick plasma 25-OH D_3. Chicks hatched from broiler breeders at 31-33, 46-48 and 61-63 wk of age. The Control treatment were chicks from broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 were chicks from broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. A) 1 d post-hatch. Significant interaction of broiler breeder age and diet. B) 4 d post-hatch. There was no significant interaction therefore, means separation based on significant age effect only. Plasma samples were obtained and measured for 25-OH D_3 by HPLC. Significant differences ($P<0.0001$) between means are indicated with a differing lowercase letter.

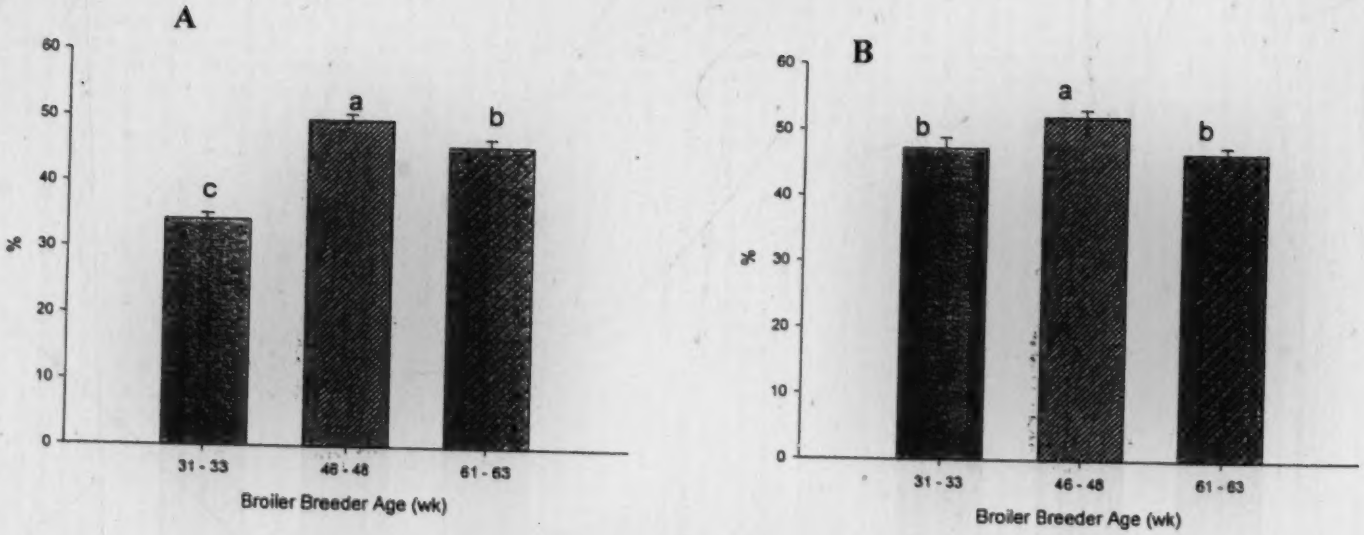


Figure 7-6. The effect of broiler breeder age on chick leukocyte % phagocytosis. Chicks hatched from broiler breeders at 31-33, 46-48 and 61-63 wk of age. Broilers breeders were on one of two treatments; Control treatment were fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 were fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. Peripheral blood leukocytes from male chicks were assessed for % phagocytosis by the percentage of leukocytes engulfing at least one fluorescent *E. coli* as measured by flow cytometry. Fluorescence of non-phagocytosing cells were determined from a control sample to which no fluorescent bacteria was added. A) 1 d post-hatch. B) 4 d post-hatch. Significant differences ($P<0.0001$) between means are indicated with a differing lowercase letter.

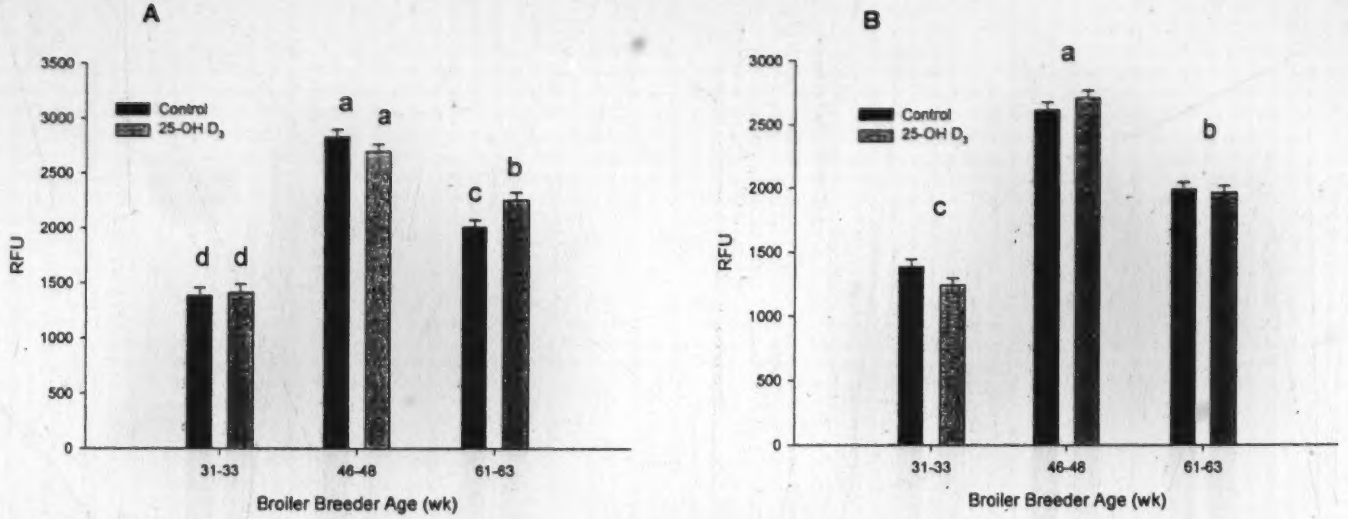
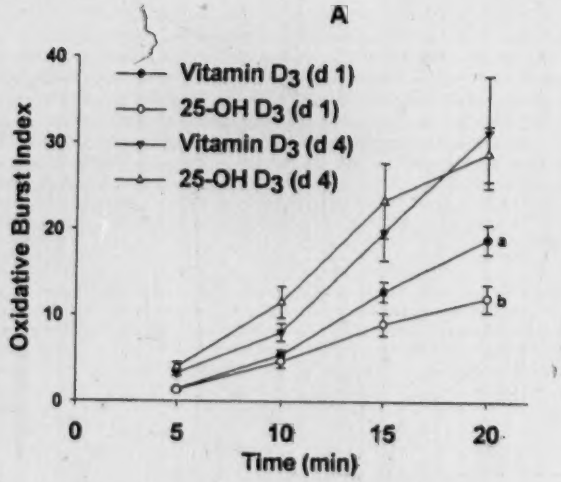
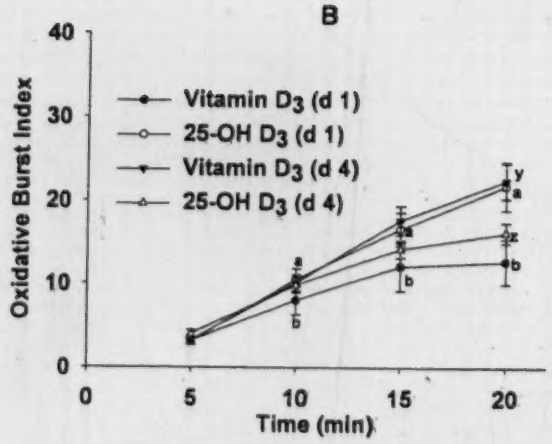


Figure 7-7. The effect of broiler breeder age on the amount number of *E. coli* phagocytosed per chick leukocyte. Chicks hatched from broiler breeders at 31-33, 46-48 and 61-63 wk of age. Broilers breeders were on one of two treatments; Control treatment were fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 were fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. A) 1 d post-hatch. Significant interaction of broiler breeder age and diet. B) 4 d post-hatch. There was no significant interaction therefore, means separation based on significant age effect only. Peripheral blood leukocytes from male chicks were assessed for phagocytocytic ability using fluorescent *E. coli* as measured by flow cytometry as relative fluorescent units (RFU). Fluorescence of non-phagocytosing cells were determined from a control sample to which no fluorescent bacteria was added. Significant differences ($P<0.0001$) between means are indicated with a differing lowercase letter.





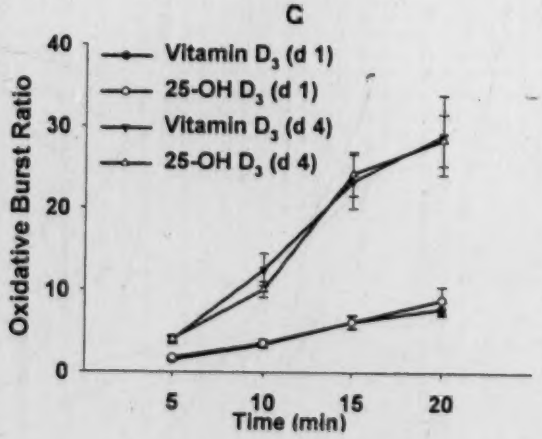


Figure 7-8. Effect of maternal vitamin D source on oxidative burst response of broiler chicks at 1 and 4 d post-hatch. A) Chicks hatched from broiler breeders at 31 to 33 wk. B) Chicks hatched from broiler breeders at 45 to 47 wk. C) Chicks hatched from broiler breeders at 61 to 63 wk. The Control treatment were the chicks from broiler breeders fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 treatment were the chicks from broiler breeders fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. Peripheral blood from males chicks was assessed for the strength of heterophil oxidative burst response over 20 minutes, measured as the ratio of increase in fluorescence from non-stimulated heterophils from the same chick. Significant differences ($P<0.0001$) between means with the same chick age are indicated with a differing lowercase letter.

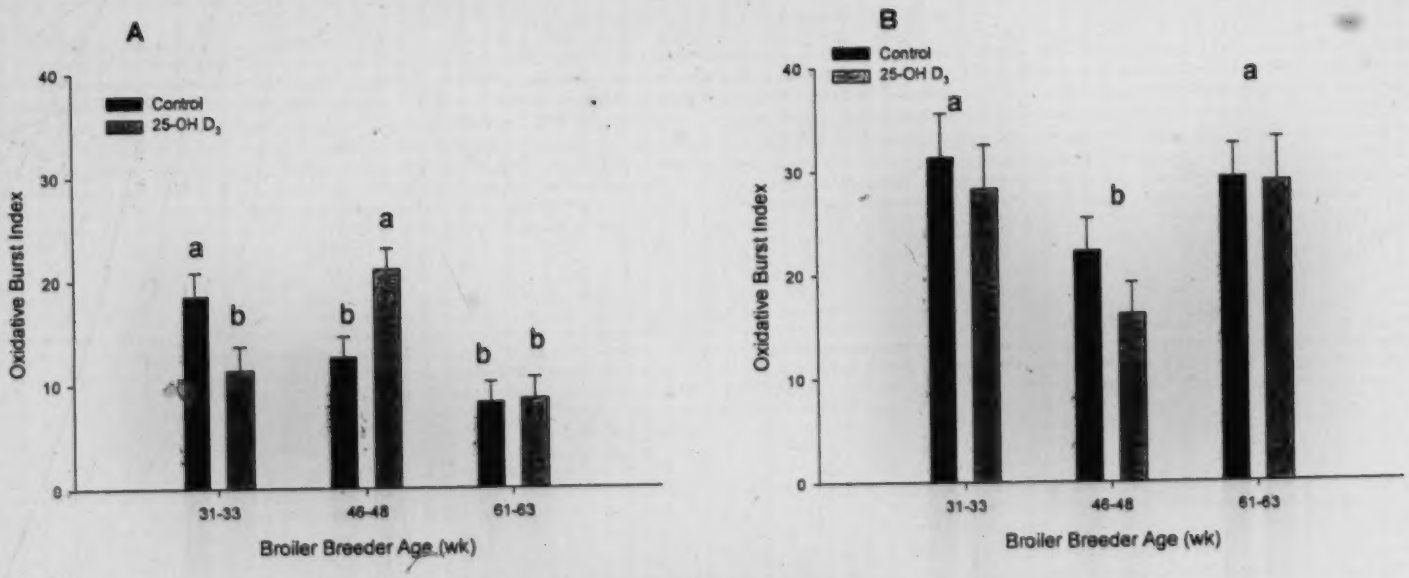


Figure 7-9. The effect of broiler breeder age on chick leukocyte oxidative burst. Chicks hatched from broiler breeders at 31-33, 46-48 and 61-63 wk of age. Broilers breeders were on one of two treatments; Control treatment were fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 were fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. A) 1 d post-hatch. Significant interaction of broiler breeder age and diet. B) 4 d post-hatch. There was no significant interaction therefore, means separation based on significant age effect only. Peripheral blood leukocytes from male chicks were assessed for oxidative burst at stimulation with PMA and measuring fluorescence intensity by flow cytometry. Oxidative burst index is a ratio of fluorescence after stimulation divided by fluorescence of non-stimulated cells from the same chick. Significant differences ($P<0.0001$) between means are indicated with a differing lowercase letter.

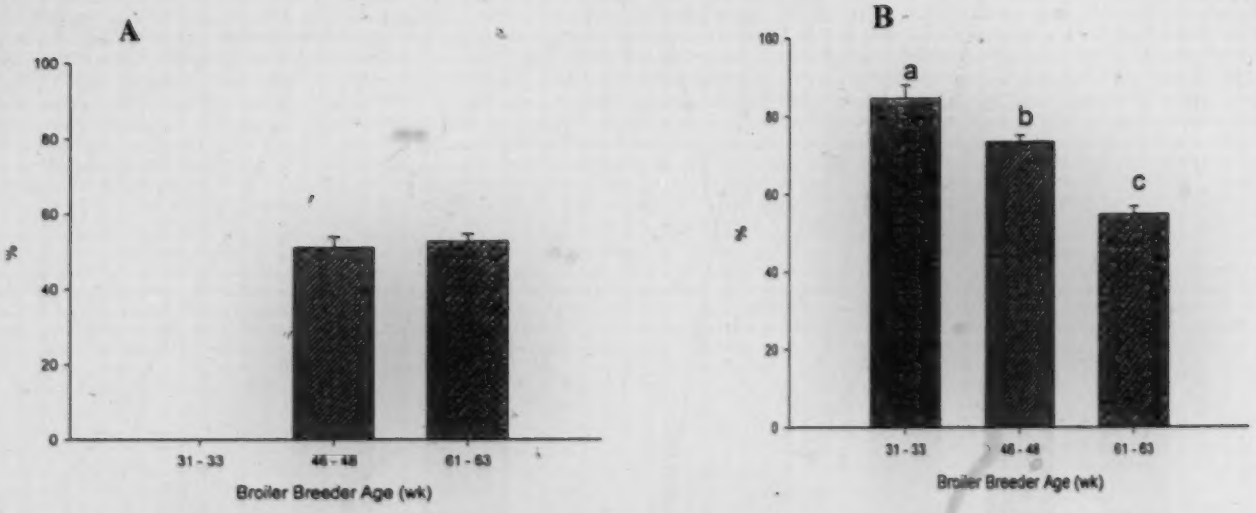


Figure 7-10. The effect of broiler breeder age on chick leukocyte bactericidal capability. Chicks hatched from broiler breeders at 31-33, 46-48 and 61-63 wk of age. Broilers breeders were on one of two treatments; Control treatment were fed a diet containing 2,760 IU of vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. The 25-OH D_3 were fed a diet containing 69 µg of 25-OH vitamin D_3 as the sole supplemental source of vitamin D activity from 23 to 64 wk of age. Peripheral blood leukocytes from male chicks were assessed for total % killing of *E. coli* after 90 min incubation. A) 1 d post-hatch. B) 4 d post-hatch. Significant differences ($P<0.0001$) between means are indicated with a differing lowercase letter.

CHAPTER 8: Research Synthesis

8.1. Summary

The overall goal of the research was to first evaluate QCT as a method of assessing bone quality and poultry that would then be used in conjunction with assessing both direct and maternal supplementation of 25-OH D_3 on broiler production, bone quality and immune function. This goal was achieved in the thesis research by testing the following hypotheses:

1. **We hypothesized that QCT would provide a precise BMD measure of poultry bones.**

This hypothesis was addressed in Chapter 2 of the thesis and includes a secondary objective, where we examined the effect of bone handling treatments, which are commonly employed prior to BMD analysis, on QCT BMD and cross-sectional area. This hypothesis was supported by the results in chapter 2, which indicated that QCT does provide precise measures of BMD and cross-sectional area, especially for the cortical and total measures of BMD and cross-sectional area. In addition, bone handling treatment had a significant effect on BMD and cross-sectional area measures.

2. **We hypothesized that QCT BMD and cross-sectional area would correlate with the traditional methods of bone quality evaluation.**

This hypothesis was addressed in Chapter 3, by comparing QCT BMD and cross-sectional area to traditional methods of bone quality evaluation. The results in Chapter 3, support this hypothesis as significant relationships were found between BMD and cross-sectional area measures with that of bone breaking strength, bone ash, and bone Ca. However, these relationships were fairly weak relationships due to the assessment of whole bone characteristics with the traditional methods whereas QCT examines specific bone fractions within a limited region of the bone.

3. **It was hypothesized that dietary 25-OH D_3 would enhance broiler production traits, bone quality and the inflammatory immune response as compared to dietary vitamin D_3.**

This hypothesis was addressed in Chapter 4 in which the objectives of the study were to investigate the effects of dietary 25-OH D_3 (and age at receiving dietary 25-OH D_3) on broiler production traits, plasma 25-OH D_3, bone formation and quality and carcass composition at 6 wk of age. This hypothesis was further addressed in Chapter 5 in which the objectives were to examine the effects of dietary 25-OH D_3, alone or in combination with vitamin D_3 on broiler performance, bone quality, the inflammatory response in broilers. This hypothesis is partially supported by the results in Chapters 4 and 5. Broiler production, plasma 25-OH D_3, bone quality and carcass composition were improved when broilers were fed 25-OH D_3, either for the entire production period or for the first 28 d. However broiler production performance was not different in broilers fed 25-OH D_3 at various levels, either alone or in addition to vitamin D_3 than birds fed vitamin D_3

However, 25-OH D_3, in addition to vitamin D_3 or on its own, reduced the effect of the inflammatory response on bone quality.

4. **It was hypothesized that maternal dietary 25-OH D_3 would support normal broiler breeder production, improve BMD, hatchability, progeny production performance as well as lead to a more mature innate immune system of their progeny at hatch.**

This hypothesis was addressed in Chapter 6 where the objective of the research was to investigate the effects of maternal 25-OH D_3 on fertility, hatchability, chick quality, chick production traits, plasma 25-OH D_3, and bone quality. This hypothesis was further addressed in Chapter 7 where the objectives of the study were to investigate the effects of maternal dietary 25-OH D_3 on broiler breeder production traits and BMD as well as *in vitro* innate immune function of the chicks. This hypothesis is partly supported by the results of Chapter 6 that show maternal 25-OH D_3 improved chick hatchability and lowered grower period feed conversion ratio. However, there were no improvements of chick or bone quality and little effect on chick plasma 25-OH D_3 of chicks from the maternal 25-OH D_3 treatment. The results from Chapter 7 support the hypothesis as maternal 25-OH D_3 maintained similar broiler breeder production performance (with minor improvements in egg quality), but resulted in enhanced broiler chick early innate immune function.

8.2. Introduction

Vitamin D is required for growth, health and bone development in poultry. In Canada, broiler chicken production typically takes place in light-tight, environmentally controlled barn facilities. This impairs the ultraviolet (UV) light-dependent synthesis of vitamin D that takes place in animals exposed to the sun (Norman and Hurwitz, 1993). Therefore a dietary source of vitamin D_3 is necessary for broiler production. Vitamin D is first metabolized in the liver to 25-hydroxycholecalciferol (25-OH D_3) and then in the kidney to its active form, 1,25-dihydroxycholecalciferol ($1,25(OH)_2$ D_3) (Soares, et al., 1995). Vitamin D is a vital nutrient required for several bodily processes and is primarily known for its role in Ca metabolism and therefore its requirement in bone formation and maintenance. However, more recently, vitamin D has been shown to be involved in the immune function.

A sound skeletal system is required to enable birds to move about freely to reach feed and water necessary for growth and production in commercial poultry species. The rate of bone development does not match the fast growth rates of commercial broilers (Rath, et al., 2000). Several bone development and formation problems can arise from poorly formed bones (Lilburn, 1994; Thorp, 1994; Julian, 1998), causing bone breakage, impairing the welfare of the broiler, leading to culls, as well as carcass downgrades at processing. Therefore, bone quality of broilers is of both welfare and economic concern affecting many aspects of the poultry industry, from the bird to the processors. As technology advances, new methodology for assessing bone quality arises. Quantitative CT has been used successfully in human bone biology but the recent introduction of it into poultry bone studies requires further development. This QCT technology would

allow for more indepth research into poultry bone biology with analysis of cortical and trabecular BMD and cross-sectional areas.

Birds can be exposed to pathogenic organisms as early as the initial stages of embryo development, therefore early immune system development and function is important in protecting the growing embryo and newly-hatched chick. Selection for fast growth rates of commercial broilers and turkeys has had a negative impact on the immune response, making modern birds more susceptible to infections than in the past (Bayyari, et al., 1997; Yunis, et al., 2000).

The natural hepatic production of 25-OH D_3 can become impaired (Ward, 2004; Waldenstedt, 2006). Therefore the opportunity exists to improve the vitamin D status of the chick by feeding dietary 25-OH D_3. Currently, 25-OH D_3 is commercially available for use in poultry diets under the trade name HyD® (DSM Nutritional Products, Parsippany, NJ). Providing the chick with a dietary source of 25-OH D_3 may allow for a more readily available metabolite for the conversion to $1,25(OH)_2D_3$ and the potential to enhance the functions that vitamin D metabolites serve within the body.

Therefore, the overall goals of the research in the following chapters was to first evaluate QCT as a method of assessing poultry bone quality that would then be used in conjunction with assessing both direct and maternal supplementation of 25-OH D_3 on broiler production, bone quality and immune function.

8.3. The Use of Quantitative Computed Tomography Bone Mineral Density and Cross-Sectional Area on Broiler and Broiler Breeder Tarsometatarsi

Quantitative computed tomography (QCT) uses an x-ray scan to measure bone mineral density (BMD) and cross-sectional area determined by three-dimensional distribution and densities of bone mineral. In poultry studies, the use of QCT would allow for close monitoring of changes of cortical, trabecular and total BMD and cross-sectional areas, over time that might not be apparent using measures such as total bone ash, Ca and strength measurements. Quantitative CT has only recently been introduced for use in poultry bone metabolism research (Korver, et al., 2004) and has not yet been completely validated for use in poultry. Therefore the goals of Chapters 2 and 3 were to evaluate precision of BMD and cross-sectional area as measured by QCT and to compare QCT BMD and cross-sectional area measures to traditional methods of bone quality evaluation.

8.3.1. Chapter 2. Precision of Quantitative Computed Tomography as a Tool for Assessing Bone Quality in Poultry

Quantitative computed tomography was assessed for precision for use in the study of poultry bone quality. The effect of bone handling conditions and drying on bone mineral density (BMD) of the same tarsometatarsi under live, fresh, frozen with flesh and thawed, frozen without flesh and thawed and dried treatments of six, 42 d old broilers and eight, 65 wk old broiler breeder hens. QCT precision was assessed by comparing the results from individual chicken femurs, each scanned three separate times, for each bone treatment.

Broiler cortical BMD at the midpoint was not affected by the different bone treatments. However, broiler total BMD at the midpoint and 60% distal length scans

tended to decrease with increased handling. Increased bone handling also resulted in reductions in cortical area at both the midpoint and 60% distal scans of the broiler tarsometatarsi. The broiler breeder cortical BMD at both the midpoint and 60% distal length increased with increased bone handling, whereas the opposite occurred with midpoint trabecular BMD. Broiler breeder Live measures of cortical, trabecular and total area at the mid-shaft were greater than in the other bone treatment groups.

BMD measurements of both the broiler and broiler breeder bones were more variable than bone cross-sectional area measurement for all bone treatments. Cortical and total BMD at the mid-shaft and at 60% distal length of both the broiler and broiler breeder tarsometatarsi were very repeatable within an individual bird. However, trabecular BMD of broiler and broiler breeder tarsometatarsi were more variable within each bone treatment.

The results of the current study indicate, especially for total and cortical densities and areas, that QCT allows for precise measurements of BMD and cross-sectional area in poultry. However, the trabecular BMD measure may be not accurately measured with QCT, due to the influence of the bone marrow density. In addition, consistency with regards to the scan location and the bone handling treatment within an experiment are very important when assessing poultry BMD and area using QCT.

8.3.2. Chapter 3: Validation of Quantitative Computed Tomography as a Tool for Assessing Bone Quality in Poultry

QCT was assessed as a tool for the study of poultry bone quality by determining its relationship with other commonly used bone quality methodologies. Relationships

among QCT measurements and bone breaking strength, ash and Ca were determined from one broiler breeder, one broiler and three laying hen experiments using linear regression analysis. Total BMD as measured by QCT was linearly related to bone breaking strength (BBS) of laying hen bones in all three laying hen studies (r^2=0.16 to 0.64; P<0.05) and with broiler femurs (r^2 = 0.49; P<0.05). Total BMD was also linearly related to ash weight of broiler breeder tibias (r^2=0.23; P<0.05) and ash weight, % ash and % Ca in one laying hen study (r^2=0.20, 0.22 and 0.25, respectively; P<0.05) and ash weight and % ash in another laying hen study (r^2=0.09 and 0.06, respectively; P<0.05). Total bone area was linearly related to ash weight, % ash and Ca in breeder hen tibias (r^2=0.08, 0.07 and 0.10, respectively; P<0.05) and ash weight of broiler femurs (r^2=0.17; P<0.05). Total bone area was also linearly related to BBS in two laying hen studies (r^2=0.14 to 0.35: P<0.05). Although QCT BMD and BA were linearly related to other common bone quality traits used in poultry research, the r^2-values given showed only a moderate to low relationship. This is due to the fact that QCT was used to measure a specific 1 mm-wide point along the length of the bone, whereas the other methods, such as bone ash and Ca are usually used to measure the entire bone. The comparison of BMD to strength and mineral content measures would also not be expected to have high correlations as they each measure different physiological or functional aspects of the bone. However, the fact that relationships did exist between QCT measurements at the midpoint and assessments of the whole bone indicates that QCT would be a valid tool for assessing bone quality in poultry. Under research conditions, QCT improves upon these other traditional methods by providing a more in-depth look at bone status and makes it possible to examine bone quality *in vivo*, and over time in the same bird. QCT bone

quality measures can provide a less invasive and time-consuming assessment of bone quality, while yielding a more in-depth look at bone development than other traditional methods of bone quality assessment.

8.4. 25-OH D_3 and Poultry Production

The active metabolite of vitamin D_3 is 1,25 dihydroxycholcalciferol ($1,25(OH)_2D_3$). Vitamin D_3 is hydroxylated in the liver by 25-hydroxylase to form 25-hydroxychocalciferol (25-OH D_3) (Soares, et al., 1995). This metabolite is then further hydroxylated to $1,25(OH)_2D_3$ in the kidney by 25-hydroxy-D_3-1α-hydroxylase (Norman and Hurwitz, 1993). $1,25(OH)_2D_3$ is a steroid hormone (Norman, 1968), and it is through this final metabolite that vitamin D exerts its actions on calcium metabolism and cellular differentiation (Norman and Hurwitz, 1993).

Vitamin D is required for growth, health and bone development in poultry. Vitamin D may also be involved in various aspects of the immune system. In many production environments, direct exposure to ultraviolet light is not available to the birds. This impairs the UV-dependent synthesis of vitamin D that takes place in animals exposed to the sun (Norman and Hurwitz, 1993). Therefore a dietary source of vitamin D_3 is usually necessary for birds. The overall goal of Experiments reported in Chapters 4 to 7 were to determine the effect of maternal or direct supplementation of 25-OH D_3 on the performance, bone quality and innate immune function of the broiler chick. A summary of the effects of dietary 25-OH D_3 supplementation on broiler and broiler breeder production can be found in Table 8-1.

8.4.1. Effects of Dietary 25-OH D_3 in Broilers

8.4.1.1. Chapter 4: The Effect of Dietary Vitamin D Source on Plasma 25-OH D_3, Broiler Production, Carcass Composition, and Bone Quality

Four dietary treatments were imposed. Birds were fed either vitamin D_3 or 25-OH D_3 (fed at the same, industry-relevant vitamin D activity level) from 0 to 41 d. Birds in two additional treatments were fed one of the above dietary treatments from day 0 to d 28, and switched to the opposite diet at 28 d (25-OH D_3 Early or 25-OH D_3 Late). Broilers fed 25-OH D_3 throughout the entire trial (0 to 41 d) had greater BW at 41 d than birds in the Control or 25-OH D_3 Late groups. During the grower phase, the 25-OH D_3-fed broilers gained more weight per day, which resulted in a nearly significant decrease in feed conversion ratio ($P=0.0619$). The results indicate that supplementation of the broiler diet with 25-OH D_3 can improve final broiler BW when fed for the duration of the production cycle. The birds fed 25-OH D_3 from 0 to 41 d had the greatest live BW at processing and eviscerated carcass weight. Similarly, absolute weights of the pectoralis major, wings, thighs and drums were the greatest in this treatment group. Given the higher BW in this group, these results are not surprising. However, birds from the 25-OH D_3 treatment also had a greater % of pectoralis major muscle (as a % of eviscerated carcass) and drums than the Control group ($P<0.02$).

25-OH D_3 is the most abundant circulating form of vitamin D and plasma levels of the metabolite give a good indication of the vitamin D status of the chick (Haussler and Rasmussen, 1972). Feeding dietary 25-OH D_3 at a concentration of 69 µg/kg feed increased circulating levels of this metabolite within the broiler chicken. At all time

points measured (10, 28 and 41 d), the broilers consuming dietary 25-OH D_3 had significantly greater plasma levels of this metabolite than those fed IU vitamin D_3. Also of interest was the lack of a decrease of 25-OH D_3 in the plasma from 0 to 10 d of age in the chicks fed 25-OH D_3 as occurred among the broilers fed vitamin D_3. These results indicate an impairment of conversion of dietary vitamin D_3 to 25-OH D_3 between 0 and 10 d of age. The greater 25-OH D_3 plasma levels in the birds fed dietary 25-OH D_3 than vitamin D_3, may indicate either a difference in the absorption of these nutrients from the gut or quite possibly impairment in the hydroxylation of vitamin D to 25-OH D_3 in the liver.

Leg problems are a major welfare and economic concern for the broiler industry (Rath, et al., 2000). The most well-known function of vitamin D_3 within the chick is its involvement in calcium metabolism and therefore its involvement in bone development and maintenance. Bone quality of broilers is of both welfare and economic concern affecting many aspects of the poultry industry, from the bird to the processors. The results from Chapter 4 show that dietary 25-OH D_3 resulted in greater femur breaking strength, femur BMD and cross-sectional area at various ages throughout the broiler production cycle. Bone issues are a serious problem that the broiler industry is currently battling. Providing a nutritional supplement as a way of combating these issues may increase feed cost but could potentially reduce bird losses due to leg problems, reduce culls and/or trims at processing and improve meat yield. In addition, although difficult to measure in terms of dollars, improving bird welfare could also improve the image of poultry production to the public.

Overall, 25-OH D_3 fed throughout the entire production period or from 0 to 28 d was able to increase plasma 25-OH D_3 and resulted in a more efficient growth during the grower period (10 to 28 d), improved BMD and cross-sectional area, bone strength and increased breast muscle yield. Although fed at similar levels of vitamin D_3 activity, dietary 25-OH D_3 was able to increase important broiler production parameters relative to the vitamin D_3 and the 25-OH D_3 Late treatments. Therefore, 25-OH D_3, may confer additional benefits as compared to vitamin D_3, fed at the same level of activity, especially when available to the bird during the first 28 d post-hatch or throughout the entire production period.

8.4.1.2. Chapter 5: The Effect of Dietary Vitamin D Source on Broiler Inflammatory and Antibody Immune Responses and Bone Quality

Two series of diets were formulated. One series of experimental diets contained vitamin D_3 at 2,500 IU/kg of feed plus 25-OH D_3 added at either 0 (D), 17.25 (D+ 25HD), 34.5 (D+ 50HD), 69 (D+100HD) or 103.5 (D+150HD) µg/kg of diet, which reflect 0, 25, 50, 100, and 150% of the manufacturer-recommended levels of 25-OH D_3, respectively. A second series of diets contained the same levels of 25-OH D_3 in the absence of vitamin D_3, for a total of 9 diets. At 11 and 13 d of age, 16 birds per dietary treatment were selected at random, injected with *Salmonella typhimurium* lipopolysaccharide (LPS) to simulate an infectious challenge (Korver, et al., 1998). There were no effects of vitamin D source or level on broiler production performance from 0 to 42 d of age. There were no differences due to dietary treatment on overall body weight gain from 11 to 14 d of age for the bird selected for the LPS portion of the

experiment. However, the LPS-injected birds had reduced BW following the LPS injections as a result of a slower rate of BW gain than the non-injected birds during this time. Femur total and trabecular BMD, total and cortical cross-sectional areas, bone weight, bone length and bone breaking strength of broilers at 14 d (4 d after LPS injection) were not different among any of the dietary treatments. However, femur cortical BMD of broilers at 14 d was greatest for those birds which received either D+100HD or 150HD. This may indicate a benefit for 25-OH D_3 during this critical bone formation period. However bone breaking strength at 42 d was nearly greater for the birds fed the 100HD diet among the birds injected with LPS at 11 and 14 d of age ($P<0.08$).

During an inflammatory challenge dietary 25-OH D_3 at 69 µg/kg + 2500IU vitamin D_3, or at 103.5 µg/kg alone reduced the effect of LPS on bone development. Therefore, 25-OH D_3 may be helpful, in addition to vitamin D_3 or on its own, at reducing potential bone issues related to an inflammatory response when birds are challenged with a bacterial infection.

8.4.2. Effect of vitamin D Source and Broiler Breeder Performance

The role of vitamin D in Ca metabolism makes it an especially important nutrient for egg-laying poultry. Previous work has shown that the vitamin D_3 level in the maternal diet is positively correlated with the vitamin D_3 and 25-OH D_3 content within the egg yolk (Mattila, et al., 1999). The early development of vitamin D metabolism within the embryo signifies the importance of this nutrient to *in ovo* development (Turner, et al., 1987).

8.4.2.1. Chapter 6: The Effect of Maternal Dietary Vitamin D Source on Fertility, Hatchability, Chick Quality and Progeny Growth and Bone Mineral Density

Broiler breeders at a commercial farm were fed dietary vitamin D_3; half of the birds also received 25-OH D_3 supplementation in the water for the duration of the breeder phase. The addition of 25-OH D_3 to the water of the breeders resulted in a greater proportion of eggshell on the egg than those eggs from breeders that received only dietary vitamin D_3. A trend towards an improvement in hatchability of fertile eggs ($P = 0.07$) was found as a result of a significant ($P < 0.03$) 33% reduction in early embryonic mortality due to the broiler breeder supplementation of 25-OH D_3. This indicates that 25-OH D_3 has some sort of protective effect on the developing embryo from 0 to 7 d of incubation. This would improve the return to the producer by resulting more viable chicks per broiler breeder. Supplementation of broiler breeder diets with 25-OH D_3 in the water in addition to 3,000 IU of dietary vitamin D_3 did not affect early body weight gain of the progeny, but reduced feed conversion ratio during the broiler grower phase relative to the Control group.

8.4.2.2. Chapter 7: Effect of Maternal Vitamin D Source and Early Chick Innate Immune Function

The ability of the chicken to combat a disease or infection successfully and efficiently is critical to minimize production losses. Infections often lead to poorer feed conversion, decreased BW and bone strength (Mireles, et al., 2005) as well as muscle degradation in chicks (Klasing and Johnstone, 1991; Klasing, 1998; Mireles, et al.,

2005). Therefore, even if the chick is able to successfully combat an infection in terms of survival, several economically important factors can be severely hindered. In addition, commercial broilers and turkeys are genetically selected for fast growth rate. Some reports indicate that this has had a negative impact on the immune response, making modern birds more susceptible to infections than in the past (Bayyari, et al., 1997; Yunis, et al., 2000). The group of chickens most at risk for infection and disease is the young, newly hatched chick (<1 wk of age) as various aspects of the immune system are not mature at this young age (Lowenthal, et al., 1994; Wells, et al., 1998). The abundant number of studies indicating a regulatory role for vitamin D and its metabolites within the immune system of various other species suggests the possibility of similar roles within poultry, although little research has been done to support this.

Broiler breeders were fed either vitamin D_3 or 25-OH D_3 at commercially-relevant levels as the sole source of dietary vitamin D activity. Broiler chicks were hatched at early (31-33 weeks of age), mid (46-48 weeks) and late (61-63 weeks) breeder ages. Egg production traits were measured throughout the trial. At each of the early, mid and late production ages, chicks were hatched and immune cells were isolated from peripheral blood at 1 and 4 d post-hatch. The functional activity of the cells was assessed *ex vivo*.

For the broiler breeders, 25-OH D_3 resulted in equivalent total egg production and BW as compared to vitamin D_3. Although the effects on egg traits were minimal, the small number of replicates in Chapter 7 as compared to Chapter 6 may have contributed to this. There were no differences in the percentage of immune cells phagocytising *E. coli* at any broiler breeder age. However, at the broiler breeder age of 61 to 63 wk, there was a greater number of bacteria being engulfed per immune cell thereby increasing the

potential for the bacteria to be destroyed. White blood cells of chicks from the maternal 25-OH D_3 treatment consistently killed more *E. coli* than those from the Control group at all broiler breeder and chick ages, except at d 1 post-hatch of the 46 to 48 wk broiler breeder age. Heterophil oxidative burst response was not different between the two maternal treatments except for 1 d post-hatch for the 46 to 48 wk broiler breeder age where the cells from the maternal 25-OH D_3 chicks had a greater oxidative burst response.

Overall, the immune cells of chicks from the maternal 25-OH D_3 treatment had a greater ability to kill pathogenic bacteria *ex vivo* than those of chicks from broiler breeders fed vitamin D_3. This could improve the chicks' ability to efficiently kill invading pathogens. The results indicate that the maternal 25-OH D_3 supplementation enhanced broiler chick early innate immune function. If the *ex vivo* results are consistent *in vivo*, than this may result in less resources used by the bird to fight an infection and therefore less time off feed and perhaps less tissue degradation due to the inflammatory response. Increased bactericidal activity of the 25-OH D_3 chicks at all broiler breeder and chick ages, as well as increased phagocytic and oxidative burst response at some ages measured indicate that the immune system in these young chicks is potentially more mature and better equipped to handle an infectious challenge.

8.5. Implications

Bone quality is an important concern in the poultry industry, therefore determining methods that allow for in depth bone structure analysis is crucial for accurate bone quality analysis. The use of QCT would allow for better monitoring of changes in BMD

over time that might not be apparent using measures such as total bone ash, Ca and strength measurements. The results of the current study indicate that QCT is particularly useful in determining functionally important differences in cortical BMD and cross-sectional area and structure. Cortical bone provides the majority of the bone strength therefore being able to provide a more in-depth look at this particular bone fraction would be useful in poultry bone research studies. The validation of QCT in this thesis was an important factor in understanding the role of 25-OH D_3 in bone metabolism in poultry

Direct supplementation of 25-OH D_3 (HyD) had some beneficial effects on broiler performance parameters, breast meat yield and meat quality. Bone strength and mineralization was also improved by 25-OH D_3. The lack of effect of maternal dietary vitamin D source on broiler growth and production efficiency may indicate the necessity of adding 25-OH D_3 directly in the broiler diet to result in continued effects in the offspring. However, the maternal 25-OH D_3 supplementation did result in improved ability of cells from the progeny to combat pathogenic bacteria *ex vivo* just 1 and 4 d post-hatch.

An important goal in commercial poultry production is maximum return to the producer. The health and well-being of the bird have direct effects on production efficiency of the bird. It is important to maintain production efficiency but also not to comprise on bird health and welfare. Nutrition affects the production efficiency as well as the health and welfare of the bird. The results of the current study indicate that providing vitamin D_3 in the form of 25-OH D_3, not only improved production efficiency and bone quality of broilers, but maternal supplementation also decreased embryonic

mortality during incubation, and improved early innate immune function of the progeny. Therefore this product has the potential to be a nutritional means to enhance bone quality and immune function of poultry while not compromising production efficiency.

8.6. References

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Table 8-1. Summary of results comparing the effects of dietary 25-OH D_3 supplementation on broiler and broiler breeders.

Parameter	Standard	Effect of dietary 25-OH D_3 (↑, ↓, or =)	Chapter Reference
Direct supplementation			
Broiler			
BW	D_3	↑	Chapter 4
BW	D_3	=	Chapter 5
FCR	D_3	=	Chapters 4 and 5
Plasma 25-OH D_3	D_3	↑	Chapter 4
Bone breaking strength	D_3	↑	Chapter 4
BMD and area	D_3	↑	Chapter 4
BW gain after inflammatory immune response	D_3	=	Chapter 5
Bone quality after inflammatory immune response	D_3	↑	Chapter 5
Broiler Breeder			
BW	D_3	=	Chapter 7
Settable egg production	D_3	=	Chapter 7
Bone quality	D_3	=	Chapter 7
Egg shell quality	D_3	↑[1]	Chapters 6 and 7
Plasma 25-OH D_3	D_3	↑	Chapter 7
Maternal Supplementation			
Hatchability	D_3	↑	Chapters 6 and 7
Embryonic mortality	D_3	↑	Chapters 6 and 7
Chick hatch BW	D_3	↓	Chapter 6
Chick hatch BW	D_3	↑/=	Chapter 7
Broiler BW	D_3	=	Chapter 6
Broiler FCR	D_3	↑[2]	Chapter 6
Early chick innate immunity	D_3	↑	Chapter 7

[1]Egg shell quality was only improved at 29 wk in chapter 7.
[2]FCR was only improved for the broiler grower phase (15 to 27 d).